FORM PTO-1390 (REV. 9-2001) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY 'S DOCKET NUMBER
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371	239/253WO

U.S. APPLICATION NO. (If known, see 37 CFR 1.5)
60/127,662   **09/937982**

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/US00/07981	24 March 2000	2 April 1999

TITLE OF INVENTION USE OF ASPARAGINASE AND GLUTAMINASE TO TREAT AUTOIMMUNE DISEASE AND GRAFT VERSUS HOST DISEASE

APPLICANT(S) FOR DO/EO/US
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Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.

2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.

3. [ ] This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.

4. [ ] The US has been elected by the expiration of 19 months from the priority date (Article 31).

5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [ ] is attached hereto (required only if not communicated by the International Bureau).
   b. [ ] has been communicated by the International Bureau.
   c. [X] is not required, as the application was filed in the United States Receiving Office (RO/US).

6. [ ] An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. [ ] is attached hereto.
   b. [ ] has been previously submitted under 35 U.S.C. 154(d)(4).

7. [ ] Amendments to the claims of the International Aplication under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [ ] are attached hereto (required only if not communicated by the International Bureau).
   b. [ ] have been communicated by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [X] have not been made and will not be made.

8. [ ] An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).

9. [ ] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).

10. [ ] An English lanugage translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 20 below concern document(s) or information included:

11. [ ] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.

12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

13. [ ] A FIRST preliminary amendment.

14. [ ] A SECOND or SUBSEQUENT preliminary amendment.

15. [ ] A substitute specification.

16. [ ] A change of power of attorney and/or address letter.

17. [ ] A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.

18. [ ] A second copy of the published international application under 35 U.S.C. 154(d)(4).

19. [ ] A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).

20. [ ] Other items or information:

U.S. APPLICATION NO. (if known) CFR 1.5 09/937982 INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
60/127,662 PCT/US00/07981	239/253WO

21.☐ The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):

Neither international preliminary examination fee (37 CFR 1.482)
nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO
and International Search Report not prepared by the EPO or JPO $1040.00

International preliminary examination fee (37 CFR 1.482) not paid to
USPTO but International Search Report prepared by the EPO or JPO $890.00

International preliminary examination fee (37 CFR 1.482) not paid to USPTO
but international search fee (37 CFR 1.445(a)(2)) paid to USPTO $740.00

International preliminary examination fee (37 CFR 1.482) paid to USPTO
but all claims did not satisfy provisions of PCT Article 33(1)-(4) $710.00

International preliminary examination fee (37 CFR 1.482) paid to USPTO
and all claims satisfied provisions of PCT Article 33(1)-(4) $100.00

CALCULATIONS PTO USE ONLY

ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 710.00

Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30
months from the earliest claimed priority date (37 CFR 1.492(e)). $ Ø

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	$
Total claims	15 - 20 =		x $18.00	$ Ø
Independent claims	2 - 3 =		x $84.00	$ Ø
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ $280.00	$ Ø

TOTAL OF ABOVE CALCULATIONS =	$ 710.00

☒ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above
are reduced by 1/2.   + $ 355.00

SUBTOTAL =	$ 355.00

Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30
months from the earliest claimed priority date (37 CFR 1.492(f)). $ Ø

TOTAL NATIONAL FEE =	$ 355.00

Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be
accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property + $ Ø

TOTAL FEES ENCLOSED =	$ 355.00

Amount to be refunded:	$
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a. ☐ A check in the amount of $ _____ to cover the above fees is enclosed.

b. ☒ Please charge my Deposit Account No. __12-2475__ in the amount of $ __355.00__ to cover the above fees.
A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any
overpayment to Deposit Account No. __12-2475__. A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card
information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR
1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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Michael J. Wise
NAME

37,047
REGISTRATION NUMBER

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DESCRIPTION

Use of Asparaginase and Glutaminase to Treat Autoimmune Disease and Graft Versus Host Disease

Related Applications

5   This application claims priority to U.S. Patent Application Serial No. 09/094,435, by Donald L. Durden, entitled "Utilization of *Wolinella succinogenes* asparaginase in the treatment of human hematologic and autoimmune disease" (Lyon & Lyon Docket No. 234/274), filed June 9,
10 1998, which claims priority to U.S. provisional patent application 60/049,085, filed June 9, 1997.

Field Of Invention

     The present invention relates to methods for the utilization of recombinant microbial enzymes, including
15 asparaginases and glutaminases, in the treatment of autoimmune diseases and Graft versus Host disease.

Background Of Invention

     The references cited below are not admitted to be prior art to the inventions described herein.
20   Juvenile rheumatoid arthritis (JRA) is the most common rheumatic condition of childhood. Recent long-term follow-up studies have shown that JRA is not benign and the proportion of patients with a favorable outcome is less than initially thought (Wallace, 1991; Levinson, 1992).
25 Approximately one-third of all patients achieve adequate control of their disease with nonsteroidal anti-inflammatory drugs (NSAIDs), but the remainder of patients are candidates for more aggressive therapy with second-line agents.
     Placebo-controlled trials and long-term prospective
30 studies in children with JRA showed a lack of efficacy among agents such as penicillamine, hydroxychloroquine, oral gold, and intravenous immune globulin. Brewer, 1986; Giannini,

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1993; Silverman, 1993. Secondary treatment failures even with new standard medications such as methotrexate are common, creating a high demand for new safe and effective agents in these refractory diseases.

Asparaginases are used as front-line therapy in the treatment of acute leukemia. Enzymes that deplete asparagine or glutamine possess immunosuppressive effects and have been shown to have anti-inflammatory properties. However, the mode of action and the final lethal route of susceptible cells deprived of L-asparagine or L-glutamine is still undetermined.

The clinically utilized forms of L-Asparaginase are immunogenic proteins derived either from *E. coli* (EC), *Erwinia carotovora,* or *Wolinella succinogenes* (WS). *E coli* possesses two asparaginase enzymes, one constitutive and another induced by anaerobic conditions. The asparaginase induced by anaerobic conditions is known to have a tumor inhibitory effect. Interestingly, L-Asparaginase from *E. coli* has cytotoxic, but also immunosuppressive, properties due to its glutamine depleting effect. In fact, the immunosuppressive effect of L-Asparaginase has been attributed to this glutaminase property of this enzyme. The EC asparaginase has recently been covalently modified using polyethylene glycol (PEG) conjugation, to form PEG asparaginase, to reduce antigenicity and extend the half-life of the EC enzyme.

Unlike other anti-tumor agents (cyclophosphamide, etoposide, *etc.*), asparaginases from *E. coli* (EC and EC-PEG) are not mutagenic, and not associated with second malignancy. At the same time, EC and EC-PEG enzymes are not myelosuppressive. Hence, patients treated with asparaginase are not at risk for development of sepsis or other severe life threatening conditions, for example, infections.

EC and EC-PEG have potent antileukemic activity and cause minimal toxicity in children. The limited toxicity of these enzymes is restricted to rare coagulation abnormalities in less than 1% of patients, which can be

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managed easily.   Mild allergic reactions have also been
described.

The immunosuppressive effects of EC are restricted to
its effects on the lymphoid system.   L-Asparaginase derived
5   from E. coli suppresses the humoral or cell-mediated
immunological response to T cell-dependent immunogens on
sheep red blood cells.   The EC enzyme inhibits T-cell
immunity to the antigen, SRBC, as measured by antibody
titer, ADCC, and immunoglobulin producing cells in the
10   spleen (80% reduction).   The effects of E. coli asparaginase
treatment on spleen histology and lymphocyte populations are
known to include a marked reduction in the size and
reactivity of the germinal centers, which correlates with a
marked   reduction   in   the   cytoplasmic   immunoglobulin-
15   containing cells (B-cell immunoblasts).

These data support the hypothesis that depletion of
glutamine, or asparagine together with glutamine, after
treatment with E. coli asparaginase results in marked immune
suppression.   In contrast, asparagine deprivation alone,
20   caused by the administration of the glutaminase-free
asparaginase from WS, does not affect spleen histology or
lymphocyte marker distribution and is not immunosuppressive.

Definition Of Terms

Unless otherwise expressly defined, the terms used
25   herein will be understood according to their ordinary
meaning in the art, although the following terms will be
understood to have the following meanings, unless otherwise
indicated.

An "analog" of a protein, e.g., asparaginase or
30   glutaminase, refers to a polypeptide that differs in some
way from its form(s) found naturally.   For example, in
certain embodiments, an analog of asparaginase or
glutaminase will refer to an enzyme wherein one or more
amino acids has been deleted from the naturally occurring
35   amino acid sequence.   Alternatively, one or more amino acid

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residues may be substituted with a different amino acid.
Other analogs include those wherein additional amino acids
have been added to the native sequence. For example, one or
more amino acids may be added to the amino terminus and/or
5 carboxy-terminus of the enzyme, or be inserted between
internal amino acid residues. Such analogs can be prepared
by any suitable technique, although modifying a recombinant
gene to encode the desired change(s) will typically be
employed. Other analogs include those wherein one or more
10 amino acid residues are derivatized, e.g., glycosylated,
pegylated, acylated, or otherwise bound covalently to a
molecule not attached to native form(s) of the protein. Of
course, analogs according to the invention include those
where an amino acid residue is added to or substituted in
15 the native amino acid sequence, and this new residue is
itself later modified, for example, by a covalent
modification performed after the enzyme has been at least
partially purified or isolated. Moreover, as used herein,
an asparaginase or glutaminase analog includes those that
20 have been modified and exhibit altered biochemical or
physiological properties, e.g., different substrate
specificity and/or affinity, altered quarternary structure,
etc. After generating analogs, e.g., by a rational design
strategy, random mutagenesis, etc., the proteins can be
25 screened for biological activity, as described elsewhere
herein. When large numbers of analogs are generated, high
throughput screening methods are preferred in order to
identify analogs having the desired characteristics. Those
analogs found to exhibit the desired activity in vitro may
30 then be tested in vivo for activity and pharmacokinetic
properties.

    A "unique contiguous amino acid sequence" means an
amino acid sequence not found in a naturally occurring
protein or polypeptide. Thus, a "unique contiguous amino
35 acid sequence of Wolinella succinogenes", for example,
refers to a sequence which contains one or more amino acid

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substitutions, insertions, or deletions, as compared to corresponding region of the native enzyme.

A "disease which responds to asparagine or glutamine depletion" refers to a disorder wherein the cells responsible for or otherwise correlates with the disease state either lack or have a reduced ability to synthesize, uptake, or otherwise utilize asparagine or glutamine. Depletion or deprivation of asparagine to such cells can be partial or substantially complete, so long as the desired therapeutic benefit is achieved. In certain embodiments, more than about 50% of asparagine or glutamine in the serum is depleted, preferably greater than about 75%, with depletion of more than 95% being most preferably achieved. Representative examples of diseases that respond to asparagine or glutamine depletion or deprivation include certain non-hematologic diseases. Non-hematologic diseases associated with asparagine or glutamine dependence include autoimmune diseases, for example rheumatoid arthritis, systemic Lupus erythematosus (SLE), autoimmunity, collagen vascular diseases, AIDS, etc. Other autoimmune diseases that may be treated according to the instant methods include, without limitation, osteo-arthritis, Issac's syndrome, psoriasis, insulin dependent diabetes mellitus, multiple sclerosis, sclerosing panencephalitis, systemic lupus erythematosus, rheumatic fever, inflammatory bowel disease (e.g., ulcerative colitis and Crohn's disease), primary billiary cirrhosis, chronic active hepatitis, glomerulonephritis, myasthenia gravis, pemphigus vulgaris, and Graves' disease. Notwithstanding the foregoing, any disease the cells responsible for which respond, e.g., cease proliferating, become senescent, undergo apotosis, die, etc., to asparagine or glutamine depletion may be treated in accordance with the instant methods. As those in the art will appreciate, cells suspected of causing disease can be tested for asparagine or glutamine dependence in any suitable in vitro or in vivo assay, e.g., an in vitro assay wherein the growth medium lacks asparagine or glutamine.

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A "patient" refers to an animal afflicted with a disease that responds to asparagine or glutamine depletion. Typically, patients treated in accordance with the instant methods are mammals, e.g., bovine, canine, equine, feline, ovine, porcine, and primate animals, particularly humans.

An "expression vector" refers to a nucleic acid, typically a plasmid, into which heterologous genes of interest may be cloned and subsequently expressed. For expression, such vectors are generally introduced into a suitable host cell or population of host cells. The expression vector can be introduced by any appropriate technique. Preferred techniques include transformation, electroporation, transfection, and ballistic (e.g., "gene gun") introduction. Depending upon the vector employed, suitable host cells for expression of the desired heterologous gene(s) include prokaryotic and eukaryotic cells. Preferred prokaryotic cells are transformation-competent bacterial cells such as E. coli strain and DH5α and JM 109. Preferred eukaryotic host cells include yeast and mammalian cell lines. As those in the art will appreciate, the particular expression vector/host cell system selected for expression of the desired heterologous gene depends on many factors, and is left to the skilled artisan to determine in the particular circumstances. Similarly, the conditions required for expression of the desired gene from an expression vector carrying the same depends on many factors, including the host cell type, the promoter(s) and other transcription regulation elements employed, the media (or medium) used, etc. Again, the selection made in a given circumstance is at the discretion of the artisan involved, and the particular employed is readily within the skill of such a person given the disclosure herein.

A protein that is "biologically active" is one that has at least one of the biological activities of the corresponding native protein, although the activity exhibited may differ in degree from that of the native

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protein.   For example, an analog of *W. succinogenes* asparaginase according to the invention may have a greater specific activity, longer serum half-life, *etc.* than the native form of the protein.

A protein that has an "epitope-tag" refers to a protein having one or more, preferably two or more, additional amino acids covalently attached thereto or incorporated therein. The tag has a distinct epitope that can be recognized by another protein, *e.g.*, an antibody that binds that epitope, preferably with high affinity; or a protease that cleaves in or around a specific amino acid sequence (*e.g.*, DAPI, cathepsin-C), *etc.*   For example, as used herein an "N-terminal epitope tag" can refer to a peptide attached to the N-terminus of a protein, where the peptide has a conformation recognized by a particular antibody.   Such a peptide and its corresponding antibody(ies) can be used to rapidly purify the polypeptide to which the peptide is attached by standard affinity chromatography techniques. Such antibodies, and any others used in the practice of this invention (*e.g.*, for targeting gene delivery vehicles), can be prepared used techniques widely known in the art.   For example, *see* Harlow and Lane in *Antibodies, a Laboratory Manual*, Cold Spring Harbor Laboratory, 1988.   Epitope tags may also be included at the C-terminus of the protein, and in internal regions where insertion of such a tag does not substantially and adversely affect the biological activity or pharmacokinetic properties of the enzyme.

A "therapeutically effective amount" of a protein (*e.g.*, an asparaginase, a glutaminase, or an analog thereof) means that amount required to produce the desired therapeutic effect.   Of course, the actual amount required depends on many factors, such as the disease to be treated, the progression of the disease, and the age, size, and physical condition of the patient, as discussed in more detail below.

By "altering a pharmacokinetic property of a protein" is meant that a property of a drug as it acts in the body

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over a period of time, *e.g.,* serum half-life, clearance rate, biodistribution, immunogenicity, *etc.,* is changed. Such alteration can be either an increase or decrease in the property being examined.

5   Summary Of Invention

One aspect of the present invention is directed to methods for the therapeutic utilization of native and/or recombinant forms of asparaginases and glutaminases in the treatment of diseases which respond to asparagine and/or
10   glutamine depletion, including various autoimmune diseases which respond to asparagine and/or glutamine depletion. In preferred embodiments, these methods involve administering to a patient a therapeutically effective amount of a *W. succinogenes* asparaginase or glutaminase, an analog of
15   either, or an acylated asparaginase or glutaminase derived from an organism other than *W. succinogenes.* Other asparaginases or glutaminases specifically envisioned include those from other fungal and bacterial sources, and include, but are not limited to, both recombinant and native
20   asparaginases from *Wolinella succinogenes,* and recombinant and native asparaginases/glutaminases from *E. coli, Acinetobacter,* and *Erwinia,* for example.

Representative diseases that can be treated in accordance with the instant invention include autoimmune
25   diseases, for example, arthritis (*e.g.,* rheumatoid arthritis), systemic lupus erythematosus (SLE), diabetes, and AIDS. The methods of the invention may also be used to treat Graft versus Host Disease, for example. Typically, the instant methods will be applied to humans afflicted with
30   a disease which responds to asparagine and/or glutamine depletion, although other patient classes, particularly mammals (*e.g.,* bovine, canine, equine, feline, ovine, porcine, and primate animals) suffering from a disease which responds to asparagine and/or glutamine depletion can be
35   similarly treated.

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Methods for isolating native *W. succinogenes* asparaginase, producing recombinant *W. succinogenes* asparaginase *in vitro* or *in vivo*, making derivatives, analogs, and covalent modifications thereof, and making pharmaceutical formulations therefrom were described previously in U.S. Patent application Serial No. 09/094,435, by Donald L. Durden, entitled "Utilization of *Wolinella succinogenes* asparaginase in the treatment of human hematologic and autoimmune disease" (Lyon & Lyon Docket No. 234/274), filed June 9, 1998, incorporated by reference herein in its entirety including any drawings, tables, or figures. These methods can be applied analogously to asparaginases and glutaminases from other organisms, including those from other bacterial and fungal sources, including, but not limited to, recombinant and native asparaginases/glutaminases from *E. coli*, *Acinetobacter,* and *Erwinia.*

The invention has been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein. For example, in the methods of the invention, patients can be mammals, but in some embodiments this may not include mice or rats. Similarly, although all asparaginases and glutaminases are envisioned in the methods of the invention, in some embodiments this may not include native *E. coli* asparaginase.

Other features and advantages of the invention will be apparent from the following figures, detailed description, examples, and claims.

Brief Description Of The Drawings

The present invention may be better-understood and its
advantages appreciated by those individuals skilled in the
relevant art by referring to the accompanying drawings
5 wherein:

Figure 1:   Illustrates the nucleotide sequences of the
                 forward [SEQ ID NO. 1] and reverse [SEQ ID
                 NO. 2] PCR primers used in the amplification
10   of the genomic L-asparaginase sequences of
                 W. succinogenes.

Figure 2:   Agarose gel electrophoresis of propidium
                 iodine-stained *W. succinogenes* genomic DNA
                 (lanes 1 and 2) and a 1.0 kb DNA fragment
15   derived from PCR amplification. Lanes 3 and
                 4 are DNA molecular weight markers. Lane 5
                 is the 1.0 kb *W. succinogenes*-specific PCR
                 fragment amplified using the two PCR primers
                 shown in Figure 1. Lane 6 contains a φX174
20   DNA molecular weight marker.

Figure 3:   Restriction enzyme analysis of 4 colonies
                 which were isolated following the ligation of
                 the 1.0 kb *W. succinogenes*-specific PCR
                 fragment into the PCR II vector. The 1.0 kb
25   DNA was digested with BamHl (lanes 2-5);
                 EcoRl (lanes 6-9); and BamHl and EcoRl (lanes
                 10-13). Lane 14 represents a DNA molecular
                 weight ladder. The 1.0 kb *W. succinogenes*-
                 specific DNA fragment is denoted by an arrow.

30 Figure 4:   Agarose gel electrophoresis of the DNA
                 fragments amplified from the selected,
                 "positive" clones utilizing *W. succinogenes*
                 asparaginase-specific primers. Lanes 1 and 7
                 are molecular weight markers. Lanes 2 and 4

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represent DNA extracted from bacterial
colonies #1 and #3 from lanes 2 and 4 of
Figure 3. Lane 6 represents a sample of the
W. succinogenes asparaginase PCR
5   amplification product (amplified from *W.
succinogenes* genomic DNA from Figure 2, lane
5) used in the initial ligation reaction. It
should be noted that the fragment cloned into
the PCR II vector was shown to be exactly the
10   same size (*i.e.*, 1.0 b) as the initial PCR
amplification product.

Figure 5:   Illustrates the results of a determination of
the anti-tumor activity of *W. succinogenes*
(WS), *E. coli* (EC) and *E. carotovora* (Erw)
15   asparaginases against tumors generated by the
subcutaneous injection of 6C3HED Gardner
lymphosarcoma cells in C3H mice. Anti-tumor
activity was measured as a function of
caliper-measured tumor volume (cm^3). The
20   negative control consisted of injections of
0.01 M phosphate buffer (pH 7.0) into C3H
mice using the same injection schedule as for
the asparaginases.

Figure 6:   Illustrates the DNA sequence [SEQ ID NO. 3]
25   of the modified *W. succinogenes* asparaginase-
specific DNA insert. This sequence contains
not only the coding sequence of the native *W.
succinogenes* asparaginase (beginning with
codon 40 of Figure 6 and not including the
30   final 23 3' - terminal nucleotides of Figure
6), but also 39 codons for the N-terminal
epitope "tag" shown in Figure 6.

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Figure 7:   Is a schematic representation of a chemical
                 modification for a protein, for example *W.
                 succinogenes* asparaginase.

Figure 8:   Illustrates the lack of cross-reactivity
                 between different dilutions of a patient's
                 plasma known to contain high-titer
                 neutralizing antibodies against *E. coli*
                 asparaginase and the *W. succinogenes* enzyme.

Figure 9:   Illustrates the lack of cross-reactivity
                 between different dilutions of polyclonal
                 high-titer neutralizing antibodies against *E.
                 coli* asparaginase and asparaginase derived
                 from *W. succinogenes*.

Figure 10:   Demonstrates that *E. coli* asparaginase
                 reverses established arthritis in CIA model.
                 Digital image of mouse extremity before and
                 after treatment with *E. coli* asparaginase.
                 Mice were injected with bovine collagen type
                 II in complete Freund's adjuvant on day 0 and
                 boosted with same antigen on day +21.
                 Arthritis developed on day +35 following
                 immunization (Panel A) graded as 3+ arthritic
                 involvement. Mouse treated with 50 IU of *E.
                 coli* asparaginase daily for 1 week showed
                 dramatic reversal of arthritic involvement
                 from score of +3 to 0 on day + 42 as depicted
                 in Panel B.

Figure 11:   Demonstrates the effects of *E. coli*
                 asparaginase on established arthritis in CIA
                 mouse model. CIA was induced in DBA/1 mice
                 as described above. On day +35 mice that
                 developed detectable arthritis were separated
                 into equivalent groups. One group received

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E. coli asparaginase 50 IU/injection daily
for 8 weeks the other group received PBS
injections. Arthritic scores were compiled
in blinded manner over the next 8 weeks of
evaluation as depicted in bar graphs for two
experimental groups. The data were analyzed
for statistic significance. The difference
between *E. coli* asparaginase and control PBS
treated groups on months 1 and 2 was
significant (p < 0.05).

Figure 12:   Demonstrates the effects of *E. coli*
asparaginase on established arthritis induced
LPS/CIA model. CIA was induced in DBA/1 mice
as described above.   On day +21 mice were
boosted with 100 ug collagen in Freuds
adjuvant. On day +49 and +54 we administered
LPS (40 µg/mouse IP). Mice developed LPS/CIA
on day +61 and were separated into equal
groups based on the arthritic scores. One
group was treated with *E. coli* asparaginase
50 IU daily injections IP on Monday,
Wednesday and Friday and other group was
treated with PBS. Treatment was extended to
4 weeks. The bars represent the mean
arthritic score over time. The data were
evaluated by Student t-test and the
differences observed between the *E. coli*
asparaginase-treated mice on weeks 1-4 were
statistically significant as compared to
controls at (p< 0.01).

Detailed Description Of The Invention

Asparaginases and glutaminases can be used in the
treatment of autoimmune diseases and Graft versus Host

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Disease, and alter the natural course of autoimmunity.
There is a dramatic clinical response to L-asparaginase in
cancer treatment, although host toxicity and immuno-
suppression also arise. The advantages to using L-
5   asparaginase treatment for auto-immune and Graft versus Host
diseases include the fact that immuno-suppression is a
desired effect, and that lower and less frequent doses are
likely to be required, limiting toxicity to the host.

Described herein are exemplary methodologies for the
10   isolation of "native" asparaginases and glutaminases, as
well as for the production (using recombinant expression
vectors) of recombinant asparaginases and glutaminases and
analogs thereof, e.g., those which have been acylated and
those which have been modified to include additional or
15   alternate amino acids that have been acylated or otherwise
modified (e.g., by pegylation).

The following sections elaborate upon some of the
various biochemical and physiological effects of clinical
utilization of asparaginase or glutaminase therapy in the
20   treatment of diseases associated with asparagine or
glutamine dependence.

I.   Review of the Clinical use of Asparaginase and
Glutaminase

25   Asparaginases are enzymes which catalyze the
deamidation of L-asparagine (asparaginase activity) and L-
glutamine (glutaminase activity). See Cantor, P. S. &
Schimmell, M. R., Enzyme Catalysis, 2nd ed., (T. Pettersonn
& Y. Tacashi, eds.) Sanders Scientific Press, New York pp.
30   219-23. (1990). L-glutamine serves as the amide donor in
purine biosynthesis, as well as other transamination
reactions, and hence plays a role in DNA and cyclic
nucleotide metabolism.

In vivo biochemical activity of asparaginase was first
35   documented to be present in guinea pig serum in 1922 (see
Clementi, A., La desamidation enzmatique de l'asparagine
chez les differentes especes-animals et la signification

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physiologique de sa presence dass l'organisme, 19 *Arch.*
Intern. Physiol. 369 (1922)). The subsequent discovery that
asparaginase isolated from guinea pig serum was the active
agent which inhibited the *in vivo* growth of certain
asparagine-dependent mammalian tumors without concomitant
deleterious effects on normal tissue (*see* Broome, J. D.,
Evidence that the asparaginase activity of guinea pig serum
is responsible for its anti-lymphoma effects, 191 *Nature*
1114 (1961)) suggested that this enzyme could be utilized as
an anti-neoplastic agent.

Because L-asparagine is a non-essential amino acid,
asparaginase was initially thought to represent a unique
prototype of selective chemotherapy in which treatment could
be directed specifically and selectively against asparagine-
dependent cells. However, the low levels of asparaginase in
guinea pig serum necessitated the development of a more
practical source of this enzyme.

Subsequently, microbial asparaginase isolated from
Escherichia coli and *Erwinia carotovora* were shown to act as
potent anti-leukemic agents (*see* Howard, J. B. & Carpenter,
F.H., L-asparaginase from *Erwinia carotovora*: substrate
specificity and enzymatic properties, 247 *J. Biol. Chem.*
1020 (1972); Campbell, H. A., et al., Two asparaginases from
Escherichia coli B: their separation, purification, and
anti-tumor activity, 6 *Biochemistry* 721 (1967)), and when
one of these enzymes was utilized in combination with the
chemotherapeutic agent vincristine and the corticosteroid
prednisone for the treatment of acute lymphoblastic or acute
undifferentiated human leukemia, an overall remission rate
of 93% was reported (*see* Ortega, J.A., *et al.*, L-
asparaginase, vincristine, and prednisone for the induction
of first remission in acute lymphocytic leukemia, 37 *Cancer*
Res. 535 (1977)).

While these asparaginases possess potent anti-leukemic
activity, clinical utilization of the aforementioned
microbial asparaginases resulted in a wide range of host
toxicity (*e.g.*, hepatic, renal, splenic, pancreatic

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dysfunction and blood coagulation) and pronounced immunosuppression (*see* Ohno, R. & Hersh, E. M., Immunosuppressive effects of L-asparaginase, 30 *Cancer Res.* 1605 (1970)), unlike asparaginase isolated from guinea pig
5   serum (*see* Cooney, D.A., et al., L-asparaginase and L-asparagine metabolism, 10 *Ann. Rev. Pharmacol.* 421 (1970)).

Examination of the effects of *E. coli* asparaginase treatment on spleen histology and lymphocyte populations revealed a marked reduction in both the size and reactivity
10   of the splenic germinal centers which was concomitantly associated with a marked reduction in the cytoplasmic immunoglobulin-containing cells (B-cell immunoblasts; *see* Distasio, J.A., et al., Alteration in spleen lymphoid populations associated with specific amino acid depletion
15   during L-asparaginase treatment, 42 *Cancer Res.* 252 (1982)). Additionally, examination of the lymphocyte sub-population within the spleen revealed that there was a 40% reduction in the percentage of surface immunoglobulin-expressing cells (B-cells) accompanied by an increase in the ratio of Thy-
20   1.2-expressing cells (T-cells), whereas the ratio of Lyt-2 to Lyt-1 cells remained unchanged in comparison to the control animal group. These results supported the hypothesis that glutamine, or glutamine combined with asparagine depletion initially resulting from administration
25   of *E. coli* asparaginase, caused a marked decrease in spleen lymphocytic cells of the B-cell lineage.

Another important adverse clinical effect associated with traditional microbial asparaginase treatment is hepatic dysfunction (*see* Schein, P.S., et al., The toxicity of *E.*
30   *coli* asparaginase, 29 *Cancer Res.* 426 (1969)). Patients treated with *E. coli* asparaginase generally exhibit decreased plasma levels of albumin, antithrombin III, cholesterol, phospholipids, and triglycerides. Other indications of asparaginase-induced hepatic dysfunction and
35   pathology include fatty degenerative changes, delayed bromosulfophthalein clearance, and increased levels of serum glutamic-oxaloacetic transaminase and alkaline phosphatase.

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Although some investigators have reported that low dosages of *E. coli* asparaginase result in limited hepatotoxic complications, sensitive indicators of hepatic function in some patients receiving low dosages, however, still reveals significant hepatic disease which may result in life-threatening coagulopathy (*see* Crowther, D., Asparaginase and human malignant disease, 229 *Nature* 168 (1971)).

The hepatotoxic effects of microbial asparaginases may be a result of their capability to hydrolyze both asparagine and glutamine. One biochemical difference between *E. coli* and *E. carotovora* asparaginases and the enzyme derived from guinea pig is the non-specific amidohydrolase activity associated with the microbial enzymes (*see* Howard, J.B. & Carpenter, F.H., (1972) *supra*; Campbell, H.A., et al., (1967) *supra*). For example, *E. coli* asparaginase has been shown to possess a 130-fold greater level of glutaminase activity as compared to the activity of *Wolinella succinogenes* (previously classified as *Vibrio succinogenes*) asparaginase. As a result, patients treated with the conventional microbial asparaginases show a marked reduction in serum levels of both glutamine and asparagine (*see* Schrek, R., et al., Effect of L-glutaminase on transformation and DNA synthesis of normal lymphocytes, 48 *Acta Haematol.* 12 (1972)), which may demonstrate a possible correlation between glutamine deprivation and asparaginase-induced clinical toxicity (*see* Spiers, A.D.S., et al., L-glutaminase/L-asparaginase: human pharmacology, toxicology, and activity in acute leukemia, 63 *Cancer Treat. Rep.* 1019 (1979)).

The relative importance of L-glutamine in mammalian intermediary metabolism served to stimulate further research into the possible role of glutamine deprivation in asparaginase-induced immunosuppression. Lymphoid tissue has been shown to have relatively low levels of glutamine synthetase activity (*see* El-Asmar, F.A. & Greenberg, D.H., Studies on the mechanism of inhibition of tumor growth by glutaminase, 26 *Cancer Res.* 116 (1966); Hersh, E.M., L-

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glutaminase: suppression of lymphocyte blastogenic responses
in vitro, 172 *Science* 139 (1971)), suggesting that these
tissues may be particularly sensitive to the depletion of
exogenous glutamine.   In contrast, some investigators have
proposed that asparagine depletion alone may be responsible
for asparagine-induced immunosuppression (*see* Baechtel, F.
S., *et al.,* The influence of glutamine, its decomposition
products, and glutaminase on the transformation of human
lymphocytes, 421 *Biochem. Biophys. Acta* 33 (1976)).

        While the immunosuppressive effect of *E. coli* and *E.
carotovora* asparaginases are well-documented (*see* Crowther,
D., (1971) *supra;* Schwartz, R.S., Immunosuppression by L-
asparaginase, 224 *Nature* 276 (1969)), the molecular
biological basis of these functions have not yet been fully
elucidated.   The inhibition of lymphocyte blastogenesis by
various L-glutamine antagonists (*see* Hersh, E.M. & Brown,
B.W., Inhibition of immune response by glutamine antagonism:
effect of azotomycin on lymphocyte blastogenesis, 31 *Cancer
Res.* 834 (1980)) and glutaminase from *Escherichia coli* (*see*
Hersh, E.M., (1971) *supra*) tends to be illustrative of a
possible role for glutamine depletion in immunosuppression.
It has also been demonstrated that inhibition of the
lymphoid blastogenic response to phytohemagglutinin (PHA) by
E. coli asparaginase can be reversed by the addition of L-
glutamine, but not by the addition of L-asparagine. *See*
Simberkoff, M.S. & Thomas, L., Reversal by L-glutamine of
the inhibition of lymphocyte mitosis caused by *E. coli*
asparaginase, 133 *Proc. Soc. Exp. Biol. (N. Y.)* 642 (1970).
Additionally, a correlation between immunosuppression and
the relative amount of glutaminase activity has been
suggested by the observation that *E. carotova* asparaginase
is more effective than *E. coli* asparaginase in suppressing
the response of rabbit leukocytes to PHA (*see* Ashworth,
L.A.E. & MacLennan, A.P., Comparison of L-asparaginases from
Escherichia coli and *Erwinia carotovora* as
immunosuppressant, 34 *Cancer Res.* 1353 (1974)).   However,
the significance of these *in vitro* studies is limited

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because the *in vivo* fates of asparaginases and the homeostatic control of asparagine and glutamine may result in a modification of the immunosuppressive effects of anti-neoplastic asparaginases.

Another significant problem associated with the use of microbial asparaginases is that patients treated with *E. coli* and *E. carotovora* asparaginases frequently develop neutralizing antibodies of the IgG and IgM immunoglobulin class (*see, e.g.,* Cheung, N. & Chau, K., Antibody response to *Escherichia coli* L-asparaginase: Prognostic significance and clinical utility of antibody measurement, 8 Am. J. Pediatric Hematol. Oncol. 99 (1986); Howard, J.B. & Carpenter, F.H. (1972) *supra*), which allows an immediate rebound of serum levels of asparagine and glutamine. In an attempt to mitigate both the toxic effects and immunosensitivity associated with the therapeutic utilization of *E. coli* and *E. carotovora* asparaginase, a covalently-modified *E. coli* asparaginase (PEG-asparaginase) was initially developed for use in patients who have developed a delayed-type hypersensitivity to preparations "native" of *E. coli* asparaginase (*see* Gao, S. & Zhao, G., Chemical modification of enzyme molecules to improve their characteristics, 613 *Ann. NY Acad. Sci.* 460 (1990)). However, subsequent studies established that the initial development of an immune response against *E. coli* asparaginase resulted in an 80% cross-reactivity against the PEG-asparaginase with concomitant adverse pharmacokinetic effects—neutralization of PEG-asparaginase activity and normalization of the plasma levels of L-asparagine and L-glutamine (*see* Avramis, V. & Periclou, I., Pharmodynamic studies of PEG-asparaginase (PEG-ASNase) in pediatric ALL leukemia patients, *Seventh International Congress on Anti-Cancer Treatment,* Paris, France (1997)). The development of antibodies directed against *E. coli* (EC) asparaginase and the modified PEG-asparaginase in patients is associated with neutralization of the enzymatic activity of both the EC and

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PEG-asparaginases *in vivo,* thus potentially resulting in an adverse clinical prognosis.

II.   Effects of Asparaginase Treatment on Spleen and Thymus
       Histology and Lymphocyte Population.

Examination of the effects of *E. coli* asparaginase treatment on spleen histology and lymphocyte populations shows a marked reduction in both the size and reactivity of the splenic germinal centers, and a concomitant marked reduction in the cytoplasmic immunoglobulin-containing cells (B-cell immunoblasts; *see* Distasio, J. A., *et al.* (1982), *supra*). Additionally, spleen lymphocyte sub-populations show up to a 40% reduction in the percentage of surface immunoglobulin-expressing cells (B-cells) accompanied by an increase in the ratio of Thy-1.2-expressing cells (T-cells), whereas the ratio of Lyt-2 to Lyt-1 cells remains unchanged. In contrast, asparagine deprivation alone, caused by the administration of *W. succinogenes* asparaginase, has no demonstrable effect on spleen histology or lymphocyte marker distribution.

Similarly, histological examination of the thymus following *E. coli* asparaginase administration revealed a pronounced depletion of cortical thymocytes, whereas no changes in thymus histology or cellularity were found after *W. succinogenes* asparaginase administration. Therefore, a comparison of the effects of long-term administration on spleen and thymus histology, cellularity, and weight indicated that *E. coli* asparaginase treatment was associated with a pronounced, sustained reduction in these parameters in both the spleen and thymus.

III. Covalent Modification of Asparaginases and Glutaminases

Many proteins currently used to treat human diseases have extremely short circulating half-lives which limit their efficacy. In addition, the administration of many foreign proteins (including certain recombinant proteins) is associated with allergic hypersensitivity responses which

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can also lead to the production of neutralizing antibodies
which hasten the rapid elimination of these therapeutic
proteins from plasma. To overcome these and other problems,
the invention provides a covalent modification procedure to
chemically modify proteins, including asparaginases and
glutaminases, in order to extend their half-lives, reduce
their immunogenicity, and increase their efficacy. This
chemical modification regimen involves the systematic
alteration of protein structures by conjugating an aliphatic
hydrocarbon chain (saturated, partially saturated, or
unsaturated, a straight chain, a branched chain, and/or a
chain of aromatic) of an acylating agent to polar groups
within the protein structure (see Figure 7). While this
process is generally applicable to any protein to be
introduced into a patient, below conditions are described
for covalently modifying *E. coli* and *W. succinogenes*
asparaginase using an acid chloride.

IV.   Compositions, Formulation, and Administration
       As described above, asparaginases and glutaminases (and
analogs and derivatives thereof) can be used to treat
diseases which respond to asparagine or glutamine depletion.
These compounds may also be used to treat such diseases
prophylactically, or to treat those patients previously
diagnosed with and treated for such a disease. For example,
a patient previously diagnosed and successfully treated
whose disease is otherwise in remission, may experience a
relapse.   Such patients may also be treated in accordance
with the claimed invention.
       Asparaginases and glutaminases, and their biologically
active analogs and derivatives, can be administered to a
patient using standard techniques.   Techniques and
formulations generally may be found in *Remington's
Pharmaceutical Sciences*, 18th ed., Mack Publishing Co.,
Easton, PA, 1990 (hereby incorporated by reference).
       Suitable dosage forms, in part, depend upon the use or
the route of entry, for example, oral, transdermal, trans-

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mucosal, or by injection (parenteral). Such dosage forms
should allow the therapeutic agent to reach a target cell or
otherwise have the desired therapeutic effect. For example,
pharmaceutical compositions injected into the blood stream
5   preferably are soluble.

Pharmaceutical compositions according to the invention
can be formulated as pharmaceutically acceptable salts and
complexes thereof. Pharmaceutically acceptable salts are
non-toxic salts present in the amounts and concentrations at
10 which they are administered. The preparation of such salts
can facilitate pharmaceutical use by altering the physical
characteristics of the compound without preventing it from
exerting its physiological effect. Useful alterations in
physical properties include lowering the melting point to
15 facilitate transmucosal administration and increasing
solubility to facilitate administering higher concentrations
of the drug. The pharmaceutically acceptable salt of an
asparaginase or glutaminase may be present as a complex, as
those in the art will appreciate.

20   Pharmaceutically acceptable salts include acid addition
salts such as those containing sulfate, hydrochloride,
fumarate, maleate, phosphate, sulfamate, acetate, citrate,
lactate, tartrate, methanesulfonate, ethanesulfonate,
benzenesulfonate, p-toluenesulfonate, cyclohexylsulfamate,
25 and quinate. Pharmaceutically acceptable salts can be
obtained from acids, including hydrochloric acid, maleic
acid, sulfuric acid, phosphoric acid, sulfamic acid, acetic
acid, citric acid, lactic acid, tartaric acid, malonic acid,
methanesulfonic acid, ethanesulfonic acid, benzenesulfonic
30 acid, p-toluenesulfonic acid, cyclohexylsulfamic acid,
fumaric acid, and quinic acid.

Pharmaceutically acceptable salts also include basic
addition salts such as those containing benzathine,
chloroprocaine, choline, diethanolamine, ethylenediamine,
35 meglumine, procaine, aluminum, calcium, lithium, magnesium,
potassium, sodium, ammonium, alkylamine, and zinc, when
acidic functional groups, such as carboxylic acid or phenol

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are present. For example, see *Remington's Pharmaceutical Sciences, supra*. Such salts can be prepared using the appropriate corresponding bases.

Pharmaceutically acceptable carriers and/or excipients can also be incorporated into a pharmaceutical composition according to the invention to facilitate administration of the particular asparaginase or glutaminase. Examples of carriers suitable for use in the practice of the invention include calcium carbonate, calcium phosphate, various sugars such as lactose, glucose, or sucrose, or types of starch, cellulose derivatives, gelatin, vegetable oils, polyethylene glycols, and physiologically compatible solvents. Examples of physiologically compatible solvents include sterile solutions of water for injection (WFI), saline solution and dextrose.

Pharmaceutical compositions according to the invention can be administered by different routes, including intravenous, intraperitoneal, subcutaneous, intramuscular, oral, topical (transdermal), or transmucosal administration. For systemic administration, oral administration is preferred. For oral administration, for example, the compounds can be formulated into conventional oral dosage forms such as capsules, tablets, and liquid preparations such as syrups, elixirs, and concentrated drops.

Alternatively, injection (parenteral administration) may be used, *e.g.*, intramuscular, intravenous, intraperitoneal, and subcutaneous injection. For injection, pharmaceutical compositions are formulated in liquid solutions, preferably in physiologically compatible buffers or solutions, such as saline solution, Hank's solution, or Ringer's solution. In addition, the compounds may be formulated in solid form and redissolved or suspended immediately prior to use. For example, lyophilized forms of the asparaginase and glutaminase can be produced.

Systemic administration can also be accomplished by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the

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barrier to be permeated are used in the formulation. Such penetrants are well known in the art, and include, for example, for transmucosal administration, bile salts, and fusidic acid derivatives. In addition, detergents may be used to facilitate permeation. Transmucosal administration, for example, may be through nasal sprays, inhalers (for pulmonary delivery), rectal suppositories, or vaginal suppositories. For topical administration, compounds can be formulated into ointments, salves, gels, or creams, as is well known in the art.

The amounts of the active therapeutic agent to be delivered will depend on many factors, including the particular therapeutic agent and the agent's IC_{50}, the EC_{50}, the biological half-life of the compound, as well as the age, size, weight, and physical condition of the patient, and the disease or disorder to be treated. The importance of these and other factors to be considered are well known to those of ordinary skill in the art. Generally, the amount of asparaginase or glutaminase to be administered will range from about 10 International Units per square meter of the surface area of the patient's body (IU/M^2) to 50,000 IU/M^2, with a dosage range of about 1,000 IU/M^2 to about 15,000 IU/M^2 being preferred, and a range of about 6,000 IU/M^2 to about 10,000 IU/M^2 being particularly preferred to treat an auto-immune disease or Graft versus Host Disease. Typically, these dosages are administered via intramuscular or intravenous injection three times per week, e.g. Monday, Wednesday, and Friday, during the course of therapy. Of course, other dosages and/or treatment regimens may be employed, as determined by the attending physician.

In addition to administering an asparaginase or glutaminase to treat a disease which responds to asparagine or glutamine depletion, other embodiments of the invention concern administration of a nucleic acid construct encoding the enzyme or an analog thereof. As those in the art will appreciate, a variety of different gene delivery vehicles (GDVs) may be employed for this purpose. GDVs include viral

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and non-viral delivery systems. Representative viral delivery systems include recombinant retroviral vectors which provide for stable, long term, and generally low level expression of one or more heterologous genes via integration in the genome of cells transfected by the virus. Here, retroviral GDVs will encode an asparaginase or glutaminase or an analog thereof, and may also include one or more other heterologous genes, for example, a gene encoding a conditionally lethal gene (e.g., thymidine kinase, which converts the pro-drug gancyclovir to its cytotoxic form) to eliminate the transfected cells, if desired.

Other viral delivery systems include those based on adeno-associated virus (AAV) and various alpha viruses, e.g., Sindbis and Venezuelan equine encephalitis virus. These other viral GDVs may provide for higher level expression, or expression for different duration, of the desired heterologous gene(s). As those in the art will appreciate, the host range for the particular virus employed may be altered by techniques well known in the art.

Non-viral GDVs useful in the practice of these embodiments of the invention include, among others, so-called "naked DNA" systems which provide the desired heterologous gene(s) in functional association with an appropriate promoter (which in certain embodiments may be an inducible or tissue-specific promoter) encoded by the nucleic acid construct. Other regulatory elements may also be included, for example, enhancers and other activators of gene expression. Preferably, such non-viral systems are incorporated into liposomes or are associated with polycationic reagents to facilitate introduction of the nucleic acid construct into cells of the patient. Of course, other components can also be included in such GDVs, e.g., molecules to target one or more particular cell types, fusogenic peptides to facilitate endocytotic vesicle escape, etc. Construction of these and other GDVs useful in the practice of this invention are within the skill of those in the art.

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Detailed Description Of The Preferred Embodiments

The following examples will serve to further illustrate various aspects of the present invention and are not intended to act in any manner as limitations on the claimed invention. In addition, methodologies are provided which will permit one of ordinary skill within the relevant arts to determine whether a derivative asparaginase or glutaminase is appropriate for utilization in the clinical therapeutic treatment of humans. For a discussion of molecular biology techniques which can be used in the practice of this invention, in addition to those described below, see Molecular Cloning, A Laboratory Manual, 2d ed., ed. Sambrook, et al., Cold Spring Harbor Laboratory Press, 1989, and Current Protocols In Molecular Biology, ed. Ausubel, et al., John Wiley & Sons, Inc., 1995.

Example 1: In Vitro Culture of W. succinogenes

W. succinogenes was grown in 10-15 liters of liquid culture media containing 0.4% yeast extract, 100 mM ammonium formate, and 120 mM sodium fumerate. The medium was adjusted to pH 7.2 prior to autoclaving. After autoclaving, a 0.2 μm filter-sterilized solution of thioglycolate was added to the room temperature culture medium to give a final concentration of 0.05%. The cultures were incubated with continuous agitation on a shaking platform in a 37°C warm-room. For large scale culture, a 500 mL pre-culture was utilized to inoculate 10-15 liters of complete culture medium.

The bacteria were collected after the cultures had reached a optical density of approximately 1.1 at a 650 nm wavelength, by centrifugation using a Sorvall high-speed continuous flow rotor. Following centrifugation, the cells were washed in a buffer containing 0.15 M sodium chloride, 0.1 M magnesium chloride, and 0.01 M mercaptoethanol. The cells were then resuspended in 0.1 M borate buffer (pH 9.0) at a final concentration of 0.5 g wet cell weight/mL borate

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buffer and stored frozen until subsequent processing for enzyme purification.

Example 2: Animals and Cell Lines

The murine model animals utilized in these experiments were Balb/C or C3H mice of 9 to 12 weeks in age (Jackson Laboratories, Bar Harbor, ME).

The therapeutic activity of L-asparaginases was determined utilizing the 6C3HED Gardner's lymphosarcoma (Gardner, W.U., *Cancer Res.*, *vol.* 4: 73 (1944)) and P1798 lymphosarcoma cell lines (ATCC) which as ascites tumors in C3H and Balb/cc mice, respectively. Alternately, the two lymphosarcoma cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum. The 6C3HED Gardner's lymphosarcoma originated in the thymus of C3H mice that were initially given high doses of estradiol. The lymphosarcoma was subsequently perpetuated by serial transplantation in the C3H mice.

W.S. asparaginase showed potent anti-tumor activity.

Example 3: Isolation of *W. succinogenes* Genomic DNA

Genomic DNA from *W. succinogenes* was extracted from bacteria grown in basal medium. Typically, bacterial cells from a 50 mL of culture were collected by centrifugation and resuspended by gentle vortexing in 1.5 mL TE buffer (pH 7.0). To the cell suspension was added 15 μL of 10% SDS to give a final concentration of 0.1% and 3 μL of a 20 mg/mL stock solution of proteinase K. The mixture was then incubated at 37°C for approximately 60 minutes, followed by several phenol/chloroform extractions. The genomic DNA was ethanol precipitated and collected by centrifugation. The *W. succinogenes* genomic DNA so isolated was sufficiently pure to use in high stringency PCR amplification.

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Example 4: PCR Amplification of *W. succinogenes* Asparaginase
Sequences

The nucleotide sequence of a 2.5 kb Hind III fragment
containing the 993 nucleotide coding region of *W.*
5   *succinogenes* asparaginase was published in 1995.   *See*
GenBank accession number X89215.   The elucidation of this
sequence facilitated the synthesis of primers specific for
PCR amplification of the gene coding, for the *W.*
succinogenes enzyme.   As illustrated in Figure 1, the
10   forward and reverse *W. succinogenes* asparaginase-specific
PCR primers forward and reverse had the following sequences:

5'-TCCGGATCCAGCGCCTCTGTTTTGATGGCT-3'   Forward PCR Primer
                                            [SEQ ID NO. 1]
                                            (BamHI] Restriction
15   Site Underlined)

5'-TGGGAATTCGGTGGAGAAGATCTTTTGGAT-3'   Reverse PCR Primer
                                            [SEQ ID NO. 2]
20   (EcoR1 Site
                                            Restriction
                                            Underlined)

It should be noted that the genomic *W. succinogenes*
asparaginase coding sequence does not naturally contain
25   either a BamH1 or EcoR1 restriction site.   However, PCR
amplification utilizing these aforementioned primers
introduced a BamH1 and EcoR1 restriction site to the 5'- and
3'-termini, respectively to facilitate directional cloning
of this amplified genomic sequence into sequencing and/or
30   expression vectors.

With respect to PCR amplification, *W. succinogenes*
genomic DNA (purified as per Example 3) was subjected to 30
cycles of PCR amplification under the following reaction
conditions: 10 µL PCR II reaction buffer; 6 µL of 25 mg/mL
35   magnesium chloride, 8 µL of 10 mM stock solutions of dNTPs,
1 µL of Taq DNA polymerase (Stratagene Corp.); 1 µL (about
50 ng) each of the *W. succinogenes* asparaginase-specific

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forward and reverse PCR primers; 1 µL of *W. succinogenes* genomic DNA; and nuclease-free PCR-grade water to bring the reaction mixture to 100 µL total volume. Following amplification, 2 µL of the PCR products were electrophoresed through a 1% agarose gel and stained with propidium iodine to assess both the specificity of the amplification reaction and the molecular weight of the resulting DNA fragments. The amplification resulted in the production of a homogeneous, 1.0 kb *W. succinogenes* asparaginase-specific DNA fragment.

Example 5: Cloning of *W. succinogenes* Asparaginase Sequences

     The amplified *W. succinogenes* asparaginase-specific amplified DNA fragment was subsequently sub-cloned into the BamH1 and EcoR1 sites of the PCRII cloning vector (Stratagene, La Jolla, CA) utilizing the following reaction conditions: 2 µL of the PCR amplified reaction products, 2 µL of the PCRII cloning vector; 1 µL of 10X ligation buffer; 4 µL of T_4 DNA ligase (Stratagene, La Jolla, CA); and distilled/deionized water to bring the total reaction volume to 10 µL. The ligation reaction was incubated at 16°C overnight and 2 µL of this reaction was utilized to transform competent *E. coli* strains DH-5α and M15.

     IPTG-induced colorimetric selection (medicated by expression of β-galactosidase in the presence of X-GAL) was utilized to identify recombinant bacterial colonies. Three white colonies (putative positive recombinants) and one blue colony (putative negative recombinants) were chosen, inoculated into a 5 mL culture of LB medium containing 100 µg/mL ampicillin, and incubated overnight at 37°C on a shaking platform. Plasmid DNA was isolated from these cultures via standard DNA "mini-prep" methodology and the DNA was dissolved in 30 µL TE buffer and digested with 3 different restriction endonucleases: BamH1; EcoR1; and BamH1/EcoR1, to ensure that the isolated plasmid DNA

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contained the expected 1.0 kb *W. succinogenes* asparaginase-specific insert.

The electrophoretic results, as illustrated in Figure 3, lanes 2 and 4, demonstrated that colonies #1 and #3 contained the expected 1.0 kb insert. To confirm that these clones contained the *W. succinogenes* asparaginase gene, the *W. succinogenes* asparaginase-specific PCR primers were used to amplify the *W. succinogenes* asparaginase-specific fragments isolated from the aforementioned clones (Figure 3, lanes 2 and 4). These primers did not mediate amplification of non-insert-containing bacterial DNA (Figure 3, lane 3). Results of this second PCR amplification demonstrated that colonies #1 and #3 contained the *W. succinogenes* asparaginase-specific DNA insert within the PCRII cloning vector, resulting in the generation of a 1.0 kb amplification product (see Figure 3, lanes 2 and 4).

The *W. succinogenes* asparaginase-specific DNA insert in the PCR II cloning vector was then removed by BamH1 and EcoR1 digestion of 10 g of plasmid DNA derived from colony #1, gel-purified via the use of Gene Clean Kit® (Stratagene, La Jolla, CA). The DNA insert was eluted from the gel with 10 μL distilled/deionized water and then ligated overnight at 16°C into the similarly restricted pGEX-2T (Amersham Pharmacia Biotech, Piscataway, N.J.) and pET-28a (Novagen, Inc., Madison, WI) vectors under the following reaction conditions: 3 μL DNA insert; 3 μL vector DNA; 4 μL 5X ligation reaction buffer; 1 μL T$_4$ DNA ligase; and 9 μL of distilled/deionized water to give a final reaction volume of 20 μL. 10 μL of each ligation reaction mixture was used to transform 50 μL of competent *E. coli* DH-5α cells. Transformants were then plated onto LB agar plates containing 100 mg/mL ampicillin. Positive transformants (*i.e.*, *W. succinogenes* asparaginase-specific DNA insert-containing transformants, pGEX-2T-WSA and pET-28-WSA, respectively) were obtained following approximately 18 hours of incubation at 37°C. To confirm that the transformants contained the *W. succinogenes* asparaginase-specific DNA

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insert, restriction endonuclease digestion using BamH1 and
EcoR1 was performed, as well as PCR amplification and DNA
sequence analysis. Results of these analyses demonstrated
that each of the selected "positive" transformants contained
5   the *W. succinogenes* asparaginase-specific DNA insert. The
nucleotide sequence of the *W. succinogenes* asparaginase-
specific DNA insert is shown in Figure 6 [SEQ ID NO. 3],
which sequence contains 117 nucleotides 5' to the initial
codes of the Wolinella gene and 23 nucleotides 3' to the
10 gene's termination codon.

Example 6: Expression of Recombinant *W. succinogenes*
Asparaginase Analogs
        To facilitate isolation of the recombinant *W.*
succinogenes (rWS) asparaginase protein, several types of
15 epitope-labeled asparaginase analogs have been constructed.
These epitope labels included: influenza hemagglutinin (HA);
glutathione-S-transferase (GST); DYLD (FLAG); and poly-
histidine (p-His). In each instance, the label is placed on
the N-terminus of the enzyme.
20   The following methodologies are utilized to isolate
these various epitope labeled rWS asparaginase proteins:

        (1) GST-sepharose (Pharmacia AB, Upsala, Sweden) column
        chromatography is utilized to purify the GST-labeled
25   rWS asparaginase enzyme expressed from the pGEX-2T-WSA
        vector, followed by cleavage by thrombin.
        (2) Protein-G-sepharose immobilized anti-HA and anti-
        FLAG antibodies (Pharmacia AB, Upsala, Sweden) is
        utilized to affinity purify the HA-or FLAG-labeled rWS
30   asparaginase enzyme.
        (3) Nickel resin (Ni-NTA [nitilo-tri-acetic acid
        resin]; Novagen, Inc., Chatsworth, CA) is used to
        affinity purify p-His-labeled rWS asparaginase enzyme.

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More specifically, for example, production of poly-histidine (p-His)-labeled, glutathione-S-transferase (GST)-rWS asparaginase requires the induction of positively transformed *E. coli* with IPTG, followed by harvesting of the bacteria (see Hochuli, E., & Dobell, N, New metal chelate absorbents selective for protein and peptide containing neighboring histidine residues, 411 *J. Chromatography* 177 (1987)). In such expression systems, vectors such as pGEX-2T and pET-28a expression vectors may be utilized to facilitate the expression of a non-epitope-labeled form of the rWS asparaginase following IPTG induction. The p-His-labeled constructs, localized in the N-terminus of the rWS asparaginase, can then be sub-cloned into the BamHl to EcoRl site of the pET-28a vector (Novagen, Inc., Chatsworth, CA) for expression of the p-His-labeled rWS enzyme.

Example 7: Purification of Native *Wolinella succinogenes* Asparaginase

The native, homotetrameric form of *W. succinogenes* asparaginase was purified according to the following methodology. *W. succinogenes* cell lysates were prepared by subjecting bacteria cultured and frozen in accordance with Example 1 to 3 to 4 freeze/thaw cycles with sonication, followed by high-speed centrifugation to remove cell debris. After centrifugation, the supernatant was brought to 0.1 M concentration of ammonium sulfate at a temperature of 4°C. The mixture was then brought to a final volume of 120% by the addition of a 2% protamine solution, followed by centrifugation for 30 min. at 21,000 x g. The supernatants were recovered, pooled, and brought to a 50% ammonium sulfate saturation and equilibrated for 30 minutes on ice with continuous stirring. The resulting solution was then dialyzed against 0.01 M potassium phosphate buffer (pH 8.0) and applied to a 3 cm x 20 cm hydroxyapatite column (prepared by: Pharmacia, Inc.) equilibrated with 0.1 M potassium phosphate buffer pH 8.0.

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The *W. succinogenes* asparaginase was eluted from the hydroxyapatite column utilizing step-wise concentrations of phosphate buffer (*i.e.*, 0.10, 0.20, 0.25, 0.30, 0.35 M phosphate buffer, pH 8.0). The eluted fractions (10 mL/fraction) were collected, assayed for asparaginase enzymatic activity, and pooled. The enzymatically-active fractions were dialyzed against 0.1 M sodium borate buffer (pH 7.0) and applied to a 3 cm x 20 cm DEAE-Sephadex column (prepared by Pharmacia, Inc.) equilibrated in 0.1 M sodium borate buffer, pH 7.0. The enzyme was eluted by use of a linear gradient of sodium chloride (0 to 1.0 M) in 0.1 M sodium borate buffer (pH 7.0). 60 mL asparaginase-containing fractions were retained. *W. succinogenes* L-asparaginase prepared utilizing this methodology has been shown to be homogeneous by SDS-PAGE electrophoresis and silver staining.

E. coli EC-2 asparaginase (Merck, Sharp & Dohme, West Point, PA) was further purified by gel filtration on Ultragel® AcA-44 (LKB Instruments, Inc., Rockville, NM). *Erwinia carotovora* asparaginase (Microbiological Research Establishment, Salisbury, England) was provided by Pharmaceutical Resources Branch of the National Cancer Institute.

Example 8: Determination of the Biochemical Characteristics of Asparaginase

The X-ray crystallographic structures of several microbial asparaginases have been elucidated (*see* Lubkowski, J. & Palm, N. (1996), *supra*). Recombinant *W. succinogenes* asparaginase which possesses acceptable clinical properties has the following characteristics: (1) catalytic activity *in vitro*, (2) preferably a native-protein-like homotetrameric structure required for functional enzymatic catalysis, and (3) with respect to the recombinant form of *W. succinogenes* asparaginase, similar to that of the native, homotetrameric form of *W. succinogenes* asparaginase, greater substrate specificity for L-asparagine and not catalyzing the

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deamidation of L-glutamine to any physiologically significant degree.

In order to quantitate the biochemical characteristics of both the native, homotetrameric and recombinant asparaginase enzymes, K_m and V_{max} enzyme kinetics, substrate specificity, pH optimum, and temperature optimum can be determined. In addition, SDS-PAGE under both reducing and non-reducing conditions, followed by silver and Coomassie Blue staining of the gels, can be utilized to establish enzyme homogeneity, evaluate subunit composition, and determine enzyme molecular weight (see Park, R. & Liu, K., A role for Shc, grb2 and raf-1 in FcR1 signal relay, 271. *J. Biol. Chem.* 13342 (1996).

The enzymatic activity of L-asparaginase can be quantitatively determined by the amount of ammonia produced upon the hydrolysis of 0.08 M L-asparagine using 0.01 M sodium phosphate buffer (pH 7.0) as the reaction buffer (see Durden, D. L. & Distasio, J. A. (1980), *supra*). The assay mixture can consist of 10 to 40 IU of a homogeneous solution of L-asparaginase enzyme diluted to 2.0 mL with 0.01 M sodium phosphate buffer (pH 7.0). Briefly, this assay system measures the deamidation of L-asparagine indirectly by quantitating the release of NH_3 as colormetrically-detected by Nessler's Reagent. A standard curve of NH_4OH may be prepared to initially derive an extinction coefficient for NH_3, based upon absorbance at 420 nm. The enzyme reaction may be initiated by the addition of the L-asparagine substrate (0.04 M). For the determination of K_m and V_{max} enzyme kinetics, a more sensitive NADPH-dependent L-asparaginase assay system can utilized (see Distasio, J. A. & Niederman, T. (1976), *supra*).

Example 9: Therapeutic Administration of Asparaginase in Murine Animal Models

The recombinant and native forms of *W. succinogenes* asparaginase may be titrated between 5 and 50 IU per

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injection and the mice can receive up to 3 daily intraperitoneal (I.P.) injections at each dose. Toxicological and pharmacological studies for the native and recombinant enzymes can be performed by the determination of serum enzyme activity (*i.e.*, serum enzyme half-life) as described in Example 8.

Example 10: Determination of Asparaginase Enzymatic Activity (Serum Half-Life)

Serum half-life determinations can be performed on Balb/c mice intraperitoneally-injected with 5 or 10 IU of native (WS) or recombinant (rWS) *Wolinelia succinogenes* asparaginase. Enzyme half-life measurements can be performed by a slight modification of a previously published procedure (*see* Durden, D. L., *et al.*, Kinetic analysis of hepatotoxicity associated with anti-neoplastic asparaginases, 43 *Cancer Res.* 1602 (1983)). Specifically, enzyme half-life measurements can be performed by obtaining a 5 µL blood sample from the tall vein of the Balb/c mice at specific intervals following the I.P. injection of the WS or rWS asparaginase. The blood samples are then kept on ice until all samples had been collected. Once sampling was completed, each 5 µL blood sample can then be immediately pipetted into 0.5 mL of cold 1.19% sodium chloride in 0.1 M sodium phosphate buffer (pH 7.0) and mixed by vigorous vortexing.

To determine serum asparaginase activity (and hence serum half-life), two 0.2 mL aliquots from each time point can be equilibrated in a 37°C water bath. The enzymatic reaction is subsequently initiated by the addition of 0.03 mL of 0.04 M L-asparagine, pre-equilibrated to 37°C prior to addition, into one of the 0.2 mL samples. The other 0.2 mL aliquot receives only 0.3 mL of distilled water and will serve as a control "blank." The substrate-containing reaction tube may be incubated at 37°C for 1 hour after which the reaction is stopped by the addition of 0.2 mL of 5% TCA.

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In addition, a 0.2 mL aliquot of 5% TCA is also added to the control "blank." The tubes are then centrifuged at 5000 x g to remove the resulting TCA-produced precipitate. Enzymatic activity may be colormetrically-determined by the addition
5 of a 0.2 mL aliquot of the substrate-containing sample to 0.2 mL of distilled water and 0.2 mL a freshly-prepared Nessler's Reagent and the absorbance at 420 nm is read using a spectrophotometer (Gilford Instrument Laboratories, Oberlin, OH).

10 Example 11: Determination of the Anti-Neoplastic Activity of Asparaginase

The anti-neoplastic (anti-lymphoma) activity of homogeneous preparation of both native (WS) and recombinant (rWS) *W. succinogenes* asparaginase, as well as that of
15 native *E. coli* (EC) and *E. carotovora* (Erw) asparaginases, can be determined utilizing the 6C3HED Gardner lymphosarcoma cell line implanted in C3H mice. This lymphoid tumor originated in the thymus of C3H mice given high doses of estradiol and was perpetuated by serial transplantation in
20 the C3H mice. In these studies, the tumor is maintained as an ascites tumor through I. P injection of 2×10^8 viable lymphosarcoma cells in 0.1 mL of PBS (pH 7.0).

To determine asparaginase anti-tumor activity, 2.5×10^6 viable 6C3HED lymphosarcoma cells from an ascites tumor is
25 injected in a volume of 0.05 mL of PBS (pH 7.0) subcutaneously in the left ventral groin of 9 to 12 week-old C3H mice. Similarly, in another series of experiments, 2.5 $\times 10^6$ viable P1798 lymphosarcoma cells from an ascites tumor is injected in a volume of 0.05 mL of PBS (pH 7.0)
30 subcutaneously in the left ventral groin of 9 to 12 week-old Balb/c mice (*see* Jack, G. W., et al., The effect of histidine ammonia-lyase on some murine tumors, 7 *Leukemia Res.* 421 (1983)). Palpable solid tumor growth generally occurred within 4 to 7 days after injection of the
35 lymphosarcoma cells. Changes in solid tumor volume are then

subsequently measured by daily caliper-based measurement of tumor dimensions along three axes. When the average tumor volume reaches 1 cm^3, intraperitoneal injection of asparaginase can be performed. A total dosage of 3 or 6 IU

5 of asparaginase may be administered in a total of six I. P injections of 0.5 or 1.0 IU asparaginase/injection, respectively. Injections may be administered twice daily for three consecutive days.

The negative control animal group receives I.P.

10 injections of 0.01 M phosphate buffer (pH 7.0) utilizing a similar injection schedule. *E. coli* and *E. carotovora* asparaginases serve as positive controls for comparison of anti-tumor activity in this series of experiments. Student's t-test will be utilized for all statistical

15 analysis of data.

Example 12: Immune Cross-Reactivity *W. succinogenes*
Asparaginase

This example describes how it was determined if antibodies in patients known to neutralize *E. coli*

20 asparaginase react with *W. succinogenes*. Specifically, an ELISA assay was performed to make this determination, as described below.

The ELISA assay was performed on two 96 well microtiter plates, as follows: asparaginase (EC on one plate, WS on the

25 other) was diluted in carbonate buffer (prepared by dissolving 1.59 g Na_2CO_3, 2.93 g $NaHCO_3$, and 0.2 g NaN_3 in 1 L of purified water; pH was adjusted to 9.0 – 9.5 using 1N HCl or 1N NaOH; the buffer was stored at 4°C for no more than two weeks before use) to a final concentration of 0.10 IU/mL.

30 54 wells on each plate were coated with 100 µL of the respective diluted asparaginase solution and incubated overnight at 4°C after being wrapped in aluminum foil to allow the enzyme to become associated with the plates.

The following morning the plates were removed, and the

35 solution from each of the wells was removed. These wells

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were then blocked with 300 µL of a 1 mg/mL solution of BSA-PBS blocking buffer, pH 7.0 (prepared fresh by adding the appropriate amount of bovine serum albumin to PBS buffer, 0.010 M sodium phosphate, pH 7.0 - 7.2, 0.9% saline). The plates were then incubated for 1 hour at room temperature. Thereafter, the plates were washed with 300 mL of saline-Tween buffer (0.145 M NaCl, 0.05% Tween 20) per well using a Dynatech Ultrawash plate washer.

The antibodies used to screen the two plates were diluted as follows: 1:100, 1:1,000; 1:2,000; 1:4,000; 1:8,000; 1:16,000; and 1:32,000. As a control, serum from a normal human patient was used. Patient serum and rabbit anti-EC asparaginase serum and normal human serum were diluted in PBS-Tween (PBS containing 0.05% Tween 20) and 100µL of each dilution was placed on each plate in triplicate according to the following grid:

CONTROL			HUMAN PATIENT			RABBIT ANTIBODIES		
1	2	3	1	2	3	1	2	3
1:1,000	1:1,000	1:1,000	1:1,000	1:1,000	1:1,000	1:1,000	1:1,000	1:1,000
1:2,000	1:2,000	1:2,000	1:2,000	1:2,000	1:2,000	1:2,000	1:2,000	1:2,000
1:3,000	1:3,000	1:3,000	1:3,000	1:3,000	1:3,000	1:3,000	1:3,000	1:3,000
1:4,000	1:4,000	1:4,000	1:4,000	1:4,000	1:4,000	1:4,000	1:4,000	1:4,000
1:8,000	1:8,000	1:8,000	1:8,000	1:8,000	1:8,000	1:8,000	1:8,000	1:8,000
1:16,000	1:16,000	1:16,000	1:16,000	1:16,000	1:16,000	1:16,000	1:16,000	1:16,000
1:32,000	1:32,000	1:32,000	1:32,000	1:32,000	1:32,000	1:32,000	1:32,000	1:32,000

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After adding the above dilutions, the plates were incubated for at least 1.5 hour at room temperature, followed by washing each plate three times with saline-Tween as described above. A 1:1,000 dilution of Horse radish
5   peroxidase-conjugated goat anti-human immunoglobulin (BioSource International) was then prepared in PBS-Tween. 100 µL of the HP-conjugated anti-human Ig was then added to each well. The plates were then covered and allowed to incubate at room temperature for 1 hour.
10   After the 1 hour incubating each plate was again washed three times with saline-Tween, as before. To detect antibody binding, 100 µL of OPD (o-phenylenediaminedihydrochloride) substrate (40 mg of OPD in 100 mL a citrate phosphate buffer (0.1M, pH 6.0, prepared by
15   combining a solution containing 13.4 g $Na_2HPO_4 \cdot 7H_2O$ (dibasic) in 500 mL distilled water with an amount of a solution containing 9.60 g citric acid (anhydrous) in 500 mL distilled water sufficient to adjust the pH to 6.0) with 334 µL of 3% H_2O_2 prepared immediately before use and kept at
20   room temperature in the dark) was added to each well and allowed to incubate at room temperature in the dark for approximately 40 minutes. The reaction in each well was stopped by adding 100 µL of 1 M phosphoric acid. The absorbance of each well was then measured at 40 nm.
25   As is shown in Figure 8, high titer neutralizing antibodies against the *E. coli* enzyme present in patient plasma failed to bind to the *Wolinella* asparaginase. This figure shows one of 6 plasma specimens collected from patients known to be allergic to the *E. coli* enzyme as well
30   as rabbit antisera raised against the E. coli asparaginase. None of these anti-*E. coli* reactive antisera bind or neutralize the *Wolinella* asparaginase activity (Figures 8 and 9). From these data it was concluded that the *W. succinogenes* enzyme is immunologically distinct from *E.*
35   *coli*, and that the *Wolinella* enzyme can be used in patients allergic to the *E. coli* enzyme (as exemplified by titration

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of patient plasma shown in Figure 8 and rabbit anti-*E. coli*
antisera shown in Figure 9).

A highly specific antisera against the *W. succinogenes*
enzyme which does not cross react with *E. coli* asparaginase
5   in Western blot analysis has also been prepared. This
reagent   is   useful   for   performing   immunological
characterizations of the native, recombinant, and various
analog forms of the *Wolinella* enzyme. Analysis of native,
recombinant,   and   analog   forms   of   *W.   succinogenes*
10 asparaginase for this type of immunologic cross reactivity
will   be   useful   in   characterization   of   genetically   and
chemically modified proteins. Importantly, these analyses
will   be   applied   to   analysis   of   clinical   specimens   during
phase I and II clinical trials of the different forms of the
15 *W. succinogenes* enzyme.

Example 13:   Methodology for Protein Modification using
Acylation.

Protein acylation is accomplished by using different
acylating   agents,   such   as   acyl   halides   (*e.g.*,   acyl
20 chlorides), carbodiimide compounds, or acid anhydrides, each
with   a   different   number   of   carbon   atoms   comprising   a
straight   or   branched   aliphatic   chain   attached   to   the
carbonyl,   or   the   modified   carbonyl   (in   the   case   of
carbodiimides),   carbon   atom.   The   acylating   agents
25 contemplated for use in practicing this invention have the
ability   to   react   with   a   polar   group   contained   within   the
peptide sequence of a protein to form an amide side chain.
The polar group is the side chain of any of the amino acids
in the primary sequence, for example, the amine group of
30 lysine or arginine, the hydroxy group of threonine, serine,
or tyrosine, or the thiol group of cysteine. Preferably,
the reaction is carried out under conditions which do not
substantially   reduce   (*i.e.*,   reduce   by   more   than   90%,
preferably less than 50%, and more preferably less than 25%)
35 the catalytic activity of the enzyme.

Briefly, the chemical reaction was started at zero time with the dropwise addition of acetyl chloride to 5,000 IU of asparaginase, derived from either *E. coli* or *W. succinogenes*, in a volume of 10 mL of 0.1 M borate buffer at pH 8.5. The final concentration of each acid chloride is 0.1 M. The chemical reaction involves a nucleophilic attack of the polar group, *e.g.*, the free amino group, within the peptide sequence of the protein, *e.g.*, asparaginase (which is maintained in an unprotonated form in the borate buffer, pH 8.5) with the reactive acylating agent. The polar group reacts with the acylating agent yielding an aliphatic hydrocarbon modified amino acid side chain. If the acylating agent is an acyl halide, an equivalent of the respective hydrohalic acid is produced. Thus, if the acylating agent is acyl chloride and the amino acid to be modified is lysine, then the reaction yields an acylated amino group and 1 equivalent of HCl (*see* Figure 7). To prevent acid conditions from destroying the structure of the protein molecule (decreasing yield of enzyme, Table 1, below), a 1 N solution of NaOH is added drop-wise to the reaction mixture every 5-10 seconds. Aliquots of 2 mL were removed at the indicated reaction times (*see* Table 1, below), and immediately dialyzed against 0.01 M phosphate buffer at pH 7.0. Protein concentration is measured by Bradford method. Enzyme activity is determined by the amount of ammonia produced upon hydrolysis of L-asparagine (0.08 M L-asparagine) with a Nessler's reagent (see Durden, D.L. et al, *Cancer Res.* 40: 1125, (1980)). Free amino groups are measured by the method of Habeeb (*see* Habeeb, A.F.S.A., *Analytical Biochemistry*, 14:328, 1966).

TABLE I

Effect of acylation with acetyl chloride on *W. succinogenes* asparaginase

	Reaction time[a](hr)	Specific Activity[b,c] (IU/mg)	Reduction of free amines[d] (%)	Recovery of Activity[c] (%)	Half-Life (hr)
Native enzyme	0	150.0	0	100.0	1.8
Derivatized enzyme	0.5	120.0	29.0	80.0	8.0
	1.0	129.0	26.8	86.0	8.2
	2.0	130.0	32.4	86.6	7.4
	3.0	120.0	30.2	80.0	7.3
	4.5	90.0	31.3	60.0	6.2

a.   The reaction is started at time 0 with the addition of acetyl chloride to 5,000 IU of *W. succinogenes* asparaginase in 10 mL of 0.1 M borate buffer, pH 8.5. Aliquots of 2.0 mL are removed at the times indicated and dialyzed against 0.01 M phosphate buffer, pH 7.0.

b.   Protein is measured in triplicate by method of Bradford.

c.   Enzyme activity is measured by determining the amount of ammonia produced upon hydrolysis of L-asparagine with Nessler's reagent.

d.   Free amino groups are measured by method of Habeeb.

Acyl modification is performed with acylating agents of different aliphatic chain lengths, e.g., a 2 carbon aliphatic chain (C2), a 4 carbon aliphatic chain (C4), a 6 carbon aliphatic chain (C6), etc. Importantly, each specific protein (e.g., asparaginase) has different numbers of free polar groups in different positions within the

protein molecule and hence each protein is optimally modified with a different length acylating agent which conjugates a different aliphatic carbon chain to the free amino groups. These include, for example, acetyl chloride (C2), butyryl chloride (C4), hexanoyl chloride (C6), decanoyl chloride (C10), as well as the use of branched chain acid chlorides including trimethyl-acetyl chloride. Also, different acylating agents may be used for different proteins. For example, with some proteins acetyl chloride may be used, whereas for other proteins acetic anhydride may be the best acylating agenst. By way of illustration, the covalent modification of the *W. succinogenes* asparaginase with the acetyl chloride is presented in Table 1.

A.   Results of Modification

There are a number of problems that have been associated with the use of enzymes for therapeutic purposes. Many of these enzymes have extremely short half-lives which severely limits their effectiveness *in vivo*. The modification of proteins using organic modification techniques of the present invention is a promising solution to many of these problems. The C2 modification of *W. succinogenes* asparaginase results in an enzyme which has a half-life of 8.2 hours in mice as compared to the 1.8 hour half-life of the native enzyme. The increase in half-life is consistent with the time course of acetylation reaction (resulting in 20-40% decrease in enzyme activity while the activity of the *W. succinogenes* asparaginase decreases with the increasing reaction time). An about 80% recovery of enzyme activity after a 30 min. reaction time was observed, a time of maximum alteration of pharmacokinetic extension of half-life to 8.0 hours. Other modification procedures which involve polymerization (*e.g.*, polyethylene-glycol modification) result in heterogenous groups of modified reaction products which may not be suitable for

administration in humans.   The acid chloride modification procedure is a systematic approach which does not yield such heterogeneity in reaction products (see Figure 7).   The greater reproducibility and more restricted nature of reaction products result in a well controlled modification of proteins and a more reliable product with predictable extension of half-life which decrease the immunogenicity, and with the advantage of being able to very carefully control the extent of modification of the polar groups present in a specific protein molecule.   Current data modifying *W. succinogenes* asparaginase demonstrate that the enzyme is modified with a C2 acylation reaction which results in the augmentation of half-life approximately four fold.   The modification of the free amino groups and the asparaginase molecule is responsible for extension of half-life.   It is suggested that the extension of half-life will correlate with a decrease in the electrostatic charge, increase in hydrophobicity and decreased immunogenicity of the *Wolinella* enzyme.   The extension of half-life and decreased immunogenicity will increase the efficacy of the *W. succinogenes* enzyme when this drug is used in the treatment of acute lymphoblastic leukemia,   autoimmune disease, or AIDS, for example, in humans.   Through this modification procedure, we are able to generate foreign proteins which have lower immunogenicity, extended half-life, and augmented efficacy. With this systematic approach of modification, any protein can be modified and the modified protein can then be used in the treatment of human disease.   Essentially, any protein that has polar groups available in its native state (essentially all known proteins) is amenable to the modification technique of the present invention.   Hence this invention extends to all proteins currently used in treatment of human, animal and plant diseases.

Example 14:   Mouse Autoimmune Disease Model

Collagen induced arthritis (CIA) in DBA/1 mice is a recognized experimental autoimmune disease model that reflects aspects of human rheumatoid arthritis. When immunized with human collagen type II, these mice develop severe arthritis with inflammation and erosions of their joints. Cellular and humoral immune mechanisms against collagen characterized by synovial proliferation and joint infiltration by inflammatory cells are believed to be involved in the pathogenesis of this arthritis model.

Susceptibility to CIA is linked to HLA class II but also requires the presence of T cells expressing variable V beta chains of their T cell receptor. Due to the T cell depleting effect of L-Asparaginase, the severity of CIA can be reduced and arthritis can be prevented (or, if initiated, the progression of the disease at least halted) by prophylactic administration of L-Asparaginase prior to immunization with collagen.

DBA/1 (H-2q) mice were purchased from Jackson Laboratories (Bar Harbour, ME), and males 8-12 weeks of age were used for immunization experiments.

A.   Induction of Arthritis

Sedated mice were immunized with 200 µg of bovine collagen type II emulsified 1:1 in complete Freud's adjuvant (CFA) (Difco, Detroit, MI) at the base of the tail. Arthritis typically developed 4-6 weeks after immunization in 60-80% of the animals. All animal manipulations were performed under ether anesthesia.

B.   Assessment of Arthritis

Arthritis of fore and hind paws was assessed using a subjective scoring system in which "0" = normal, "1" = minor swelling or erythema, "2" = pronounced, edematous swelling, and "3" = rigidity. Each limb was graded separately, giving a maximal possible score of 12 per mouse.

C.   Effect of L-Asparaginase on Existing Arthritis
(therapeutic protocol)

     At onset of arthritis symptoms, mice were treated with
5, 10, 25, or 50 IU, respectively, of EC asparaginase
intraperitoneally once a day for a total of 3 months and
compared to untreated controls.   Additional experiments
using EC-PEG and WS asparaginases can be similarly conducted
using the same outcome parameters.   WS asparaginase, which
is believed to solely deplete L-asparagine, has no known
immunosuppressive effects.   Thus, the effect of L-asparagine
depletion on the severity and prevention of arthritis can be
assessed using the WS enzyme.

     Arthritis was scored every other day for the first
month, every third day during the second month, and once a
week in the third month after onset and treatment of
arthritis symptoms.   After 3 months, mice were sacrificed
for histopathological studies.

     The data showed that _E. coli_ asparaginase has potent
anti-arthritic activity.   _E. coli_ asparaginase treatment
resulted in the reversal of pre-existing arthritis in this
model (_see_ Figures 10 and 11).   Given the recognized
correlation between this model and human disease,
asparaginase treatment should reverse, prevent, or halt the
progression of human rheumatoid arthritis and other
autoimmune states.

     Other data showed that _E. coli_ asparaginase treatment
reversed the arthritic state induced by collagen and LPS
see Figure 12).   Activity in this highly resistant form of
autoimmune arthritis confirmed the results from the mouse
model shown herein, and further supports the usefulness of
asparaginases and glutaminases in the treatment of
autoimmune diseases.   The differences in arthritic scores
between _E. coli_ treated animals and control animals were
statistically significant ($p < 0.001$).

D.   Effect of L-Asparaginase on Arthritis Prevention
(preventive protocol)

　　　　To study the ability of L-Asparaginase to prevent
arthritis, DBA/1 mice were treated I.P. with 5, 10, 25, or
50 IU, respectively, of EC asparaginase prior to
immunization (-1), parallel to immunization (0), and then at
the consecutive days 5, 10, 15, and 30 thereafter. Arthritis
was scored every other day for the first month, every third
day during the second month, and once a week in the third
and fourth month after onset of arthritis symptoms.

　　　　After four months mice were sacrificed for
histopathological studies. The administration of _E. coli_
asparaginase concomitantly with type II collagen in the DBA
mouse model completely abrogated the development of
autoimmune CIA. These results also strongly support the
role for asparaginase and/or glutaminase in the prevention
and/or treatment of autoimmune and/or Graft Versus Host
disease in humans.

E.   Assessment of Histology

　　　　Removed limbs were fixed in 10% buffered formaldehyde
for four days. After decalcification using 5% formic acid,
specimens were embedded in paraffin, cut into thin slices,
and stained for hematoxylin and eosin. Sections were
obtained from the femoro-patellar area for the knee joints
and calcaneal area for the ankle joints. Histological
parameters included the amount of inflammatory cells in the
synovial cavity and synovial tissues, amount of proteoglycan
depletion, and the destruction of articular cartilage.
Histologic specimens were interpreted by a blinded
histopathologist.

　　　　Pathologic evaluation of involved joints in _E. coli_
asparaginase-treated and control mice revealed a dramatic
difference in histopathology. Previously arthritic joints
from _E. coli_ asparaginase-treated mice demonstrated
persistence of some pannus formation, but no destruction of

joint cartilage. In contrast, joints from control mice showed massive destruction of joint cartilage and underlying bone, along with pronounced pannus and inflammatory processes.

5   Example 15:   Enzymatic and Pharmacokinetic Studies

The EC, PEG, and WS asparaginases are purified and biochemical and pharmacological analysis are performed in DBA/1 (H-2q) animals. The enzyme levels in animals treated with these asparaginases are determined in order to
10   correlate efficacy with catalytic activity.

A.   Pharmacologic Evaluation of EC, PEG, and WS in DBA/1 Mice.

Pharmacologic analysis of EC, PEG, and WS asparaginases is performed in DBA animals. Plasma L-asparagine and L-
15   glutamine is determined. Administration of asparaginase is correlated with depletion of asparagine and/or glutamine. Neutralizing antisera to EC, WS, and PEG asparaginases is used to establish a cause and effect relationship between immunosuppressive effects of PEG and WS. A WS asparaginase-
20   specific antibody is administered to mice as a negative control for EC asparaginase experiments. The *in vivo* effects of administration of neutralizing antisera to PEG and WS is correlated with plasma amino acid levels and anti-arthritic effects in the DBA mouse model (see above).
25   Enzyme half-life measurements are performed as follows: Five μL of blood from the tail vein of mice is obtained at specific time intervals after the injection of the particular asparaginase. The 5 μL blood specimen is immediately pipetted into a 0.5 mL of cold 1.19% NaCl in 0.1
30   M sodium phosphate buffer (pH 7.0) and vigorously vortexed. Blood samples are collected and kept at 4°C until all specimens are collected. For the asparaginase assay, two 0.2 mL aliquots of each time point are equilibrated to 37°C in a water bath. To start the reaction, 0.03 mL of a 0.04 M

L-asparagine solution is pipetted into one of the tubes.
The other aliquot receives 0.03 mL of distilled H_2O and serve
as a blank. The enzyme reaction is stopped after 60 minutes
incubation by pipetting 0.2 mL of 5% TCA into both the
reaction mixture and the blank. Tubes are then centrifuged
at 5000 x g, to remove precipitate. A 0.2 mL aliquot of the
supernatant is then be added to 0.2 mL of distilled H_2O, and
0.2 mL of a freshly prepared Nessler's solution is added.
Absorbance at 420 nm is determined using a spectrophotometer
(Gilford Instrument Laboratories, Oberlin, Ohio).

B.   Purification of the WS and EC Asparaginases.

       WS and EC asparaginase can be purified to homogeneity
as described by Durden, et al. in order to characterize
these enzymes and compare their biological and enzymologic
activities. PEG asparaginase is obtained from Rhone Polec
Rorer, Inc. L-asparaginase preparations are shown to be
homogeneous by SDS PAGE and free of endotoxin contamination.
The efficacy of the PEG asparaginase preparation is also
tested in these experiments.

       Biochemical analysis of the native WS, EC, and PEG
enzymes is also performed, and the Km, Vmax, and substrate
specificity of these enzymes are determined. The purity of
the enzyme preparations is established by SDS PAGE followed
by silver and Coomassie blue staining of gels.

       L-asparaginase activity is determined by the amount of
ammonia produced upon hydrolysis of L-asparagine (.08 M L-
asparagine) using a 0.01 M sodium phosphate buffer (pH 7.0)
as the reaction mixture. The assay mixture consists of 10
to 40 IU of a homogeneous enzyme solution diluted to 2.0 mL
with 0.01 M sodium phosphate buffer, pH 7.0. Briefly, this
assay measures the deamidation of asparagine indirectly by
quantitating the release of NH_3 as detected by the Nesslers
reagent. A standard curve of NH_4SO_4 is prepared in order to
derive an extinction coefficient for NH_3 based on the
absorbance at 420 nm. The enzyme reaction is initiated by

the addition of L-asparagine. For Km and Vmax enzyme kinetics, a more sensitive NADPH dependent asparaginase assay system is used.

C.   DATA ANALYSIS

Student's t-test is utilized to evaluate the observed differences between asparaginase-treated animals and control DBA animals, the effects of different asparaginase preparations, and different doses of asparaginases.

Example 16:   Asparaginase for Treatment of Graft versus Host Disease

A murine bone marrow transplant model for Graft versus Host disease (GVHD)(B6--B6D2F1) (Hill GR, et al. J Clin Invest 102:115, 1998) is used to determine if asparaginase and/or glutaminases can reverse or prevent acute or chronic form of GVHD. This involves the transfer of splenocytes and lymph node cells isolated from C57BL/6J mice to F1 progeny of C57BL/6J x DBA/2J mouse breeding (termed B6D2F1), resulting in bone marrow transplantation across MHC and minor H antigen barriers. In this model, parameters of survival, spleen index, histopathology of liver, skin, small intestine, lung, and spleen are measured with cr without asparaginase/glutaminase treatment. This model has shown predictive value in testing agents for treatment of clinically significant GVHD (Kelemen E, et al. Int Arch Allergy Immunol 102:309, 1993).

For these experiments, 13-16 week B6D2F1 mice are irradiated 1300 cGy total body radiation split into two fractions 3 hours apart (^{137}Cs Source). These mice serve as recipients of 60×10^6 splenocytes and lymph node cells from C57BL/6J mouse administered by tail vein injection in 0.3 mL of HBSS on day 0 as described (Ellison CA, et al. J Immunol 155:4189, 1995; Ellison CA, et al. J Immunol 161:631, 1998). Mice are monitored daily for toxicity, body weight, and evidence of GVHD. Mice are treated on day +1 with *Wolinella*

or *E coli* asparaginase (50 IU/injection, on Monday, Wednesday, and Friday) for 4 weeks duration.

In another experimental group, mice are treated at time of onset of GVHD with a similar regimen of asparaginase or glutaminase. Splenomegaly associated with GVHD in these mice is monitored in a subset of mice by monitoring total body weight of mice and determining spleen weight. A splenic index (SI) is determined as shown below and spleens are submitted for histopathological analysis.

$$SI = \frac{\text{Spleen wt. (experimental)}}{\text{Total body weight (experimental)}} \qquad \frac{\text{Spleen wt (control)}}{\text{Total body weight (control)}}$$

Pathological analysis includes examination of H and E stained paraffin-embedded sections of liver, spleen, skin, kidney, lungs, and small intestine for lymphoid infiltration and inflammatory damage to tissues. These are graded according to a histopathological scale as described (Kelemen E, et al. Int Arch Allergy Immunol 102:309, 1993), hereby incorporated by reference herein, including any figures, drawings, or tables. *E. coli* asparaginase can ameliorate the severity of acute GVHD in this model.

While embodiments and applications of the present invention have been described in some detail by way of illustration and example for purposes of clarity and understanding, it would be apparent to those individuals whom are skilled within the relevant art that many additional modifications would be possible without departing from the inventive concepts contained herein. The invention, therefore, is not to be restricted in any manner except in the spirit of the appended claims.

All references cited herein are hereby incorporated in their entirety. When used above, the term "including" means "including, without limitation," and terms used in the

singular shall include the plural, and vice versa, unless the context dictates otherwise.

Claims:

1. Method of preventing or treating an autoimmune disease that responds to asparagine or glutamine depletion, said method comprising the step of administering to a human patient having said autoimmune disease a therapeutically effective amount of an asparaginase or a glutaminase.

2. A method according to claim 1, wherein said asparaginase is selected from the group consisting of *E. coli*, *Wolinella succinogenes*, and *Erwinia* asparaginases.

3. A method according to claim 2, wherein said asparaginase is recombinant.

4. A method according to claim 2, wherein said asparaginase is native.

5. A method according to claim 1, wherein said glutaminase is *Acinetobacter* glutaminase.

6. A method according to claim 5, wherein said glutaminase is recombinant.

7. A method according to claim 5, wherein said glutaminase is native.

8. A method according to claim 1, wherein said autoimmune disease is selected from the group consisting of rheumatoid arthritis, systemic lupus erythematosus, and diabetes.

9. Method of preventing or treating Graft versus Host Disease, said method comprising the step of administering to a human patient having said Graft versus Host Disease a therapeutically effective amount of an asparaginase or a glutaminase.

10. A method according to claim 9, wherein said asparaginase is selected from the group consisting of *E. coli, Wolinella succinogenes,* and *Erwinia* asparaginases.

11. A method according to claim 10, wherein said asparaginase is recombinant.

12. A method according to claim 10, wherein said asparaginase is native.

13. A method according to claim 9, wherein said glutaminase is *Acitenobacter.*

14. A method according to claim 13, wherein said glutaminase is recombinant.

15. A method according to claim 13, wherein said glutaminase is native.

PCT

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(54) Title: USE OF ASPARAGINASE AND GLUTAMINASE TO TREAT AUTOIMMUNE DISEASE AND GRAFT VERSUS HOST DISEASE

(57) Abstract

Described herein are methods for using asparaginases and/or glutaminases to treat patients with asparagine and/or glutamine–dependent diseases. Some diseases specifically envisioned to be treated by the methods of the invention include Graft versus Host disease and auto–immune diseases, including SLE, RA, diabetes, and AIDS. The glutaminases and asparaginases can be native or recombinant, and can be modified by acylation or pegylation, for example.

Figure 1: <u>Nucleotide sequences of the forward and reverse PCR primers used in the amplification of the genomic L-asparaginase sequences of <i>Wolinella succinogenes</i>.</u>

[SEQ ID NO. 1] - Forward PCR Primer (BamH1 Site Underlined)

5'-TCC<u>GGATCC</u>AGCGCCTCTGTTTTGATGGCT-3'

[SEQ ID NO. 2] - Reverse PCR Primer (EcoR1 Site Underlined)

5'-TGG<u>GAATTC</u>GGTGGAGAAGATCTTTTGGAT-3'

Figure 2: Agarose gel electrophoresis of propidium iodine-stained *Wolinella succinogenes* genomic DNA and a 1.0 Kb PCR fragment
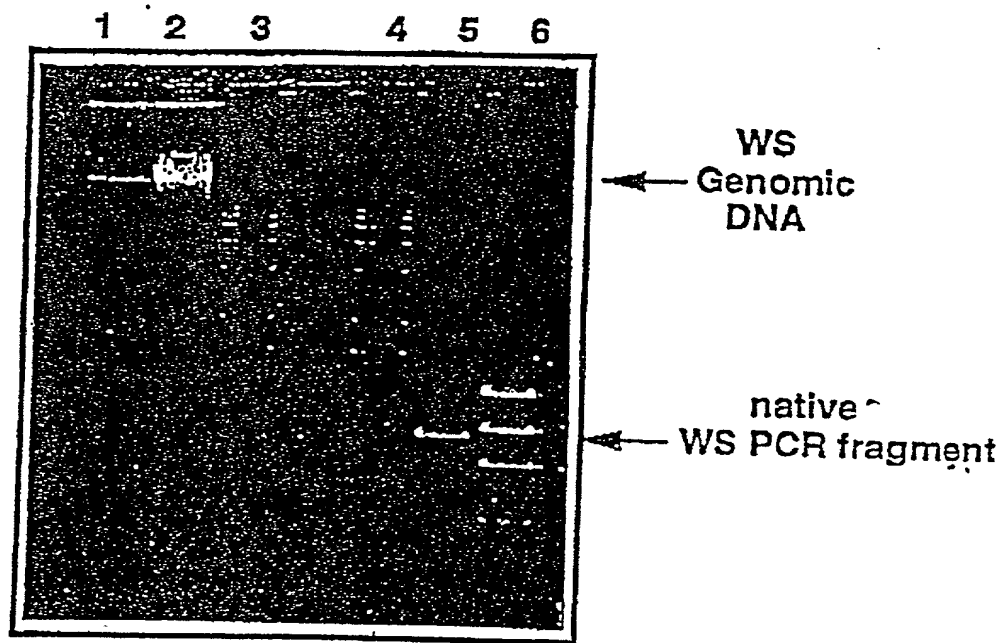
Figure 3: <u>Restriction enzyme analysis of 4 colonies which were isolated following the ligation of the 1.0 Kb *Wolinella succinogenes*-specific PCR fragment into the PCR II vector</u>
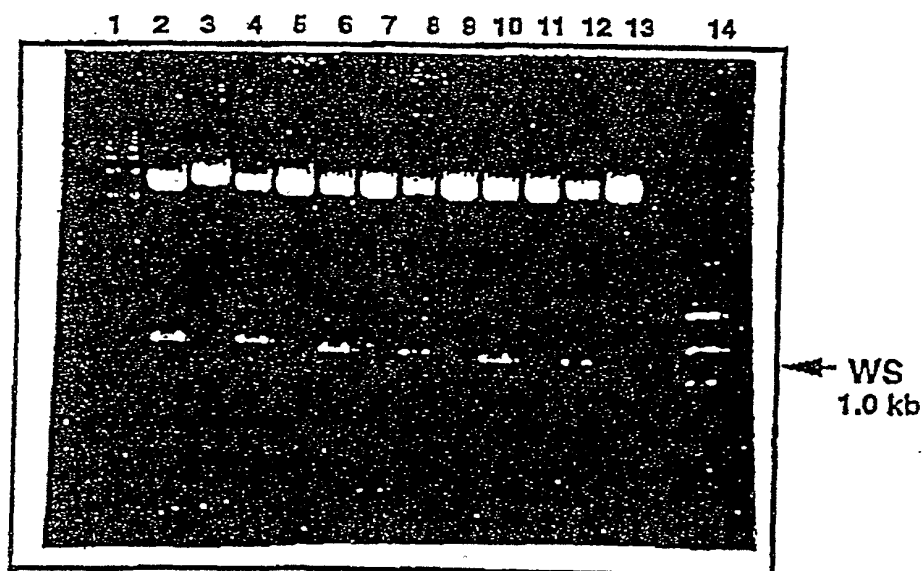
Figure 4: <u>Agarose gel electrophoresis of the DNA fragments amplified from the selected, "positive" clones utilizing <i>Wolinella succinogenes</i> asparaginase-specific primers</u>
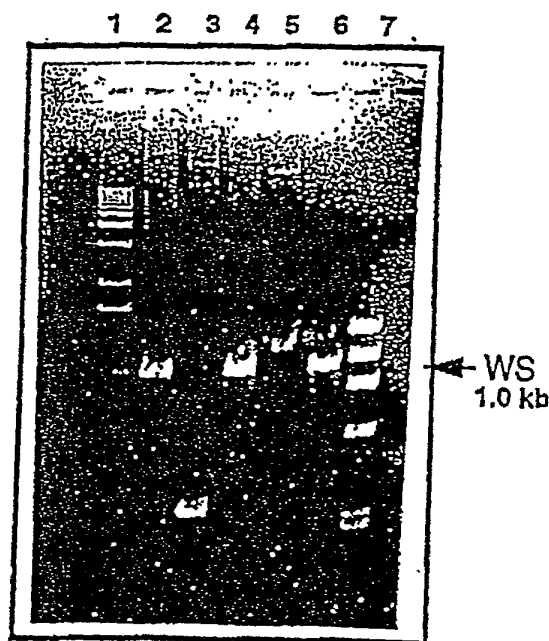
Figure 5: Determination of the anti-tumor activity of *Wolinella succinogenes*
(WS), *Escherichia coli* (EC), and *Erwinia carotovora* (Erw)
asparaginases against tumors generated by the injection of 6C3HED
Gardener's lymphosarcoma cells in C3H mice
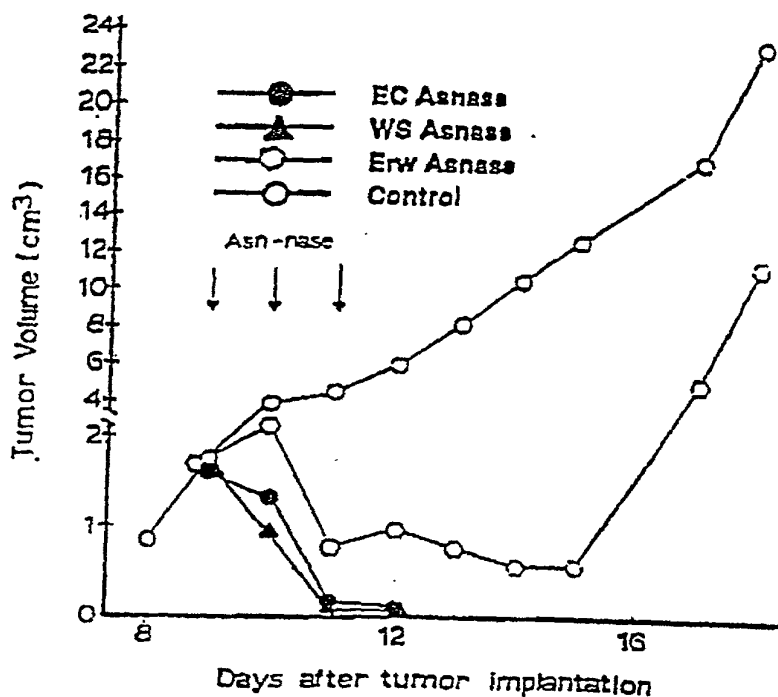
Figure 6: DNA sequence of the modified *Wolinella succinogenes* asparaginase-specific recombinant cDNA insert

```
ATG GGC AGC AGC CAT CAT CAT CAT CAT CAT AGC AGC GGC CTG GTG CCG
CGC GGC AGC CAT ATG GCT AGC ATG ACT GGT GGA CAG CAA ATG GGT CGC
GGA TCC AGC GCC TCT GTT TTG ATG GCT AAA CCC CAA GTG ACT ATC CTA
GCC ACA GGA GGC ACC ATC GCT GGT TCG GGG GAA TCT AGC GTC AAG AGT
AGC TAC TCT GCT GGA GCA GTC ACC GTT GAT AAG CTT CTT GCA GCC GTC
CCT GCC ATC AAC GAC CTA GCC ACC ATC AAG GGT GAA CAG ATC TCA AGC
ATT GGC TCC CAA GAG ATG ACG GGT AAG GTG TGG CTT AAA CTA GCC AAG
CGT GTC AAT GAG CTC CTC GCC CAA AAA GAG ACC GAA GCC GTG ATC ATC
ACC CAT GGA ACT GAC ACC ATG GAA GAG ACC GCT TTC TTC CTC AAC CTC
ACG GTG AAA AGC CAA AAA CCT GTC GTC CTT GTA GGC GCC ATG CGT CCA
GGC TCT TCC ATG AGT GCT GAT GGC CCC ATG AAT CTC TAT AAC GCC GTG
AAT GTA GCG ATC AAC AAA GCC TCT ACT AAC AAA GGA GTG GTG ATT GTG
ATG AAC GAT GAG ATT CAC GCC GCC AGA GCG ACC AAG CTC AAC ACC
ACC GCA GTC AAT GCA TTT GCT TCG CCC AAC ACA GGT AAA ATC GGC ACA
GTC TAT TAT GGC AAA GTC GAG TAT TTC ACT CAA TCC GTT CGA CCT CAC
ACC CTT GCA AGT GAG TTT GAT ATT AGC AAA ATC GAA GAA CTC CCC AGA
GTC GAT ATT CTT TAC GCT CAC CCC GAT GAT ACT GAT GTT TTA GTC AAT
GCA GCC CTT CAG GCA GGA GCC AAA GGA ATC ATC CAT GCA GGC ATG GGC
AAT GGG AAC CCT TTC CCT TTG ACT CAA AAT GCT CTT GAA AAA GCA GCC
AAA TCA GGC GTA GTC GTC GCT CGA AGC TCT AGA GTG GGC AGT GGT TCC
ACC ACC CAA GAG GCT GAA GTG GAT GAT AAG AAA CTT GGT TTT GTG GCT
ACA GAG AGT CTC AAC CCT CAA AAA GCC AGA GTG CTT CTT ATG TTA GCC
CTC ACC AAA ACT AGT GAT AGA GAG GCG ATC CAA AAG ATC TTC TCC ACC
TAT TAA TCCAAGAAAGGGAATCTCTTCAC
```

The polyCAT sequence which encodes the polyHistidine residues, the ATG start site, and the TAA stop codon are shown in bold letters.
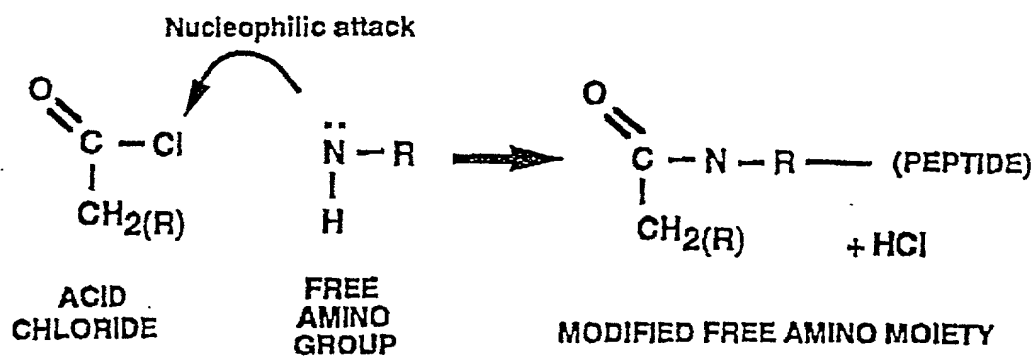
FIGURE 7

Nucleophilic attack

ACID
CHLORIDE

FREE
AMINO
GROUP

MODIFIED FREE AMINO MOIETY

pH 8.5 to maintain protonated
state of nitrogen atom.

09/937982

FIGURE   8

Patient's Antibodies Against E.coli Asparaginase Do
Not Cross React With Wolinella Asparaginase

Dillution of Patient's Plasma
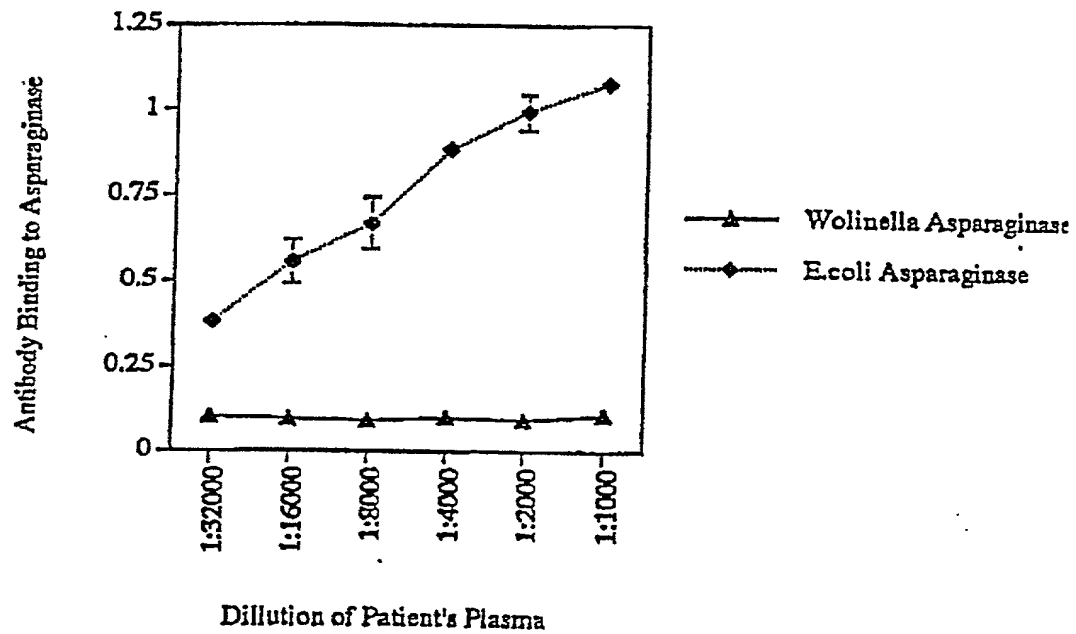
FIGURE   9

Binding of Asparaginase by Rabbit Anti-E.coli Asparaginase

FIGURE 10

A

B

09/937982
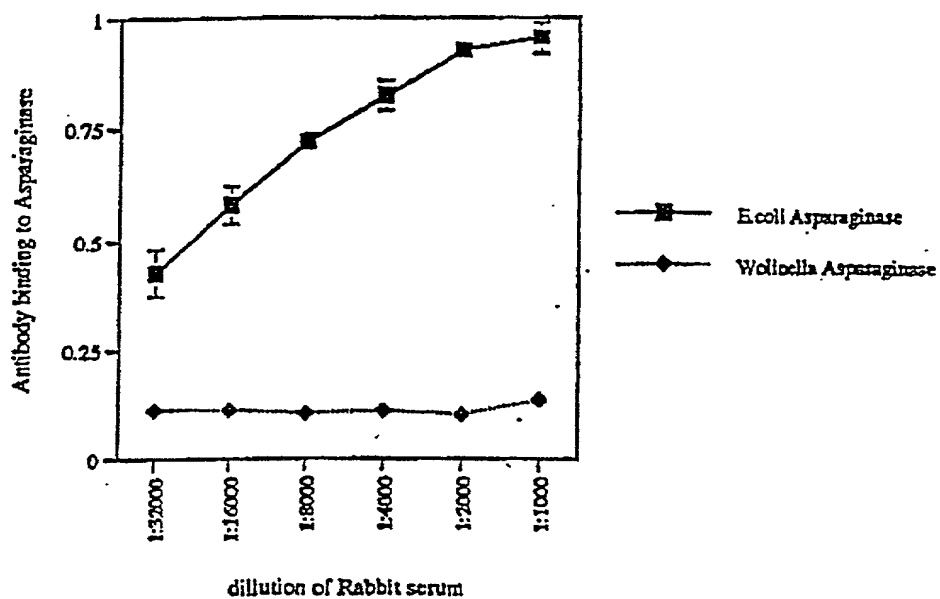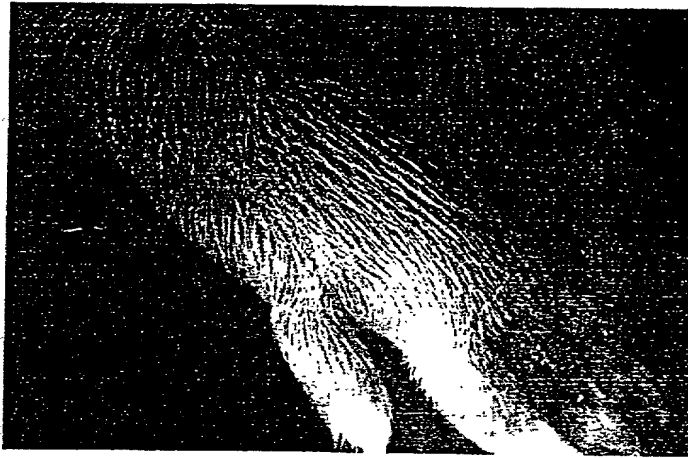
Treatm... ... of CIA DBA/1 Mice with E. ... ...i. L-Asparaginase
(Pilot Study)

FIGURE 11

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FIGURE 12

Mean Arthritic Score (MAS) for DBA/1 Mice

LPS Model
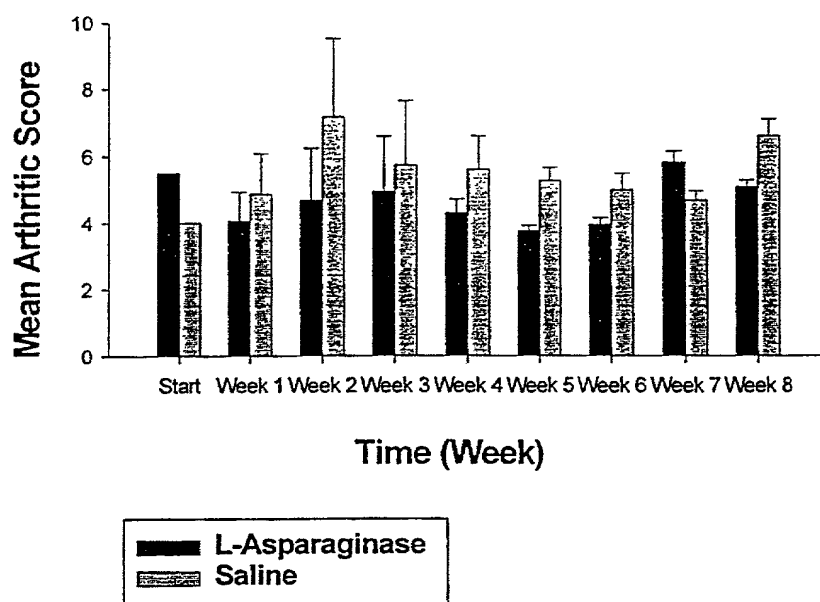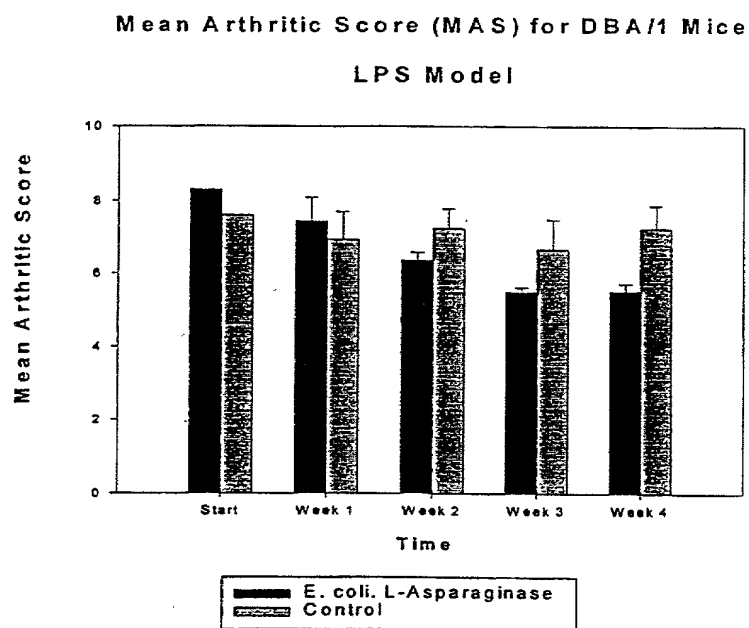
UTILITY DECLARATION
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Utility Application

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled **USE OF ASPARAGINASE AND GLULTAMINASE TO TREAT AUTOIMMUNE DISEASE AND GRAFT VERSUS HOST DISEASE** the specification of which

(Check One)

☐   is attached hereto OR

☒   was filed on <u>October 2, 2001</u> as United States Application Serial No. 09/937,982 or PCT International Application No. _____ and was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment(s) referred to above.

I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with Title 37, Code of Federal Regulations, § 1.56.

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Prior Foreign Application Number(s)	Country	Date of Filing	Priority Claimed Yes	No
PCT/US00/07981	PCT	March 24, 2000	x	

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Application Number(s)	Filing Date

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INVENTOR'S SIGNATURE _____ DATE _____

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	RESIDENCE & CITIZENSHIP	City	State or Foreign Country	Country of Citizenship	
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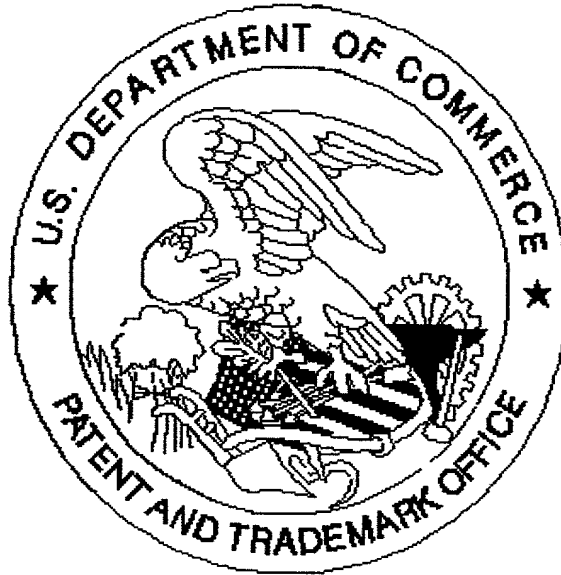
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